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This proposal is based upon the hypothesis that the protective effects of an early pregnancy and lactation on the incidence of breast cancer result from estrogen (E) and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and end bud (TEB) growth and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. The initial objective of this grant is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. Thus far, 14 clones isolated from the TEB DD-PCR fraction have been sequenced. Three clones of unknown identity are preferentially expressed in the TEB fraction. Antibodies to two other clones encoding p190-B and adrenomedullin are being used to localize and study their function. Northern blots have been used to study the expression of p190-B and adrenomedullin during mammary gland development. Procedures have been developed to isolate nuclear matrix proteins from the TEB and preliminary 2D PAGE analysis has identified several unique proteins in this fraction. Techniques have been developed to analyze cell cycle parameters including the numbers of cells in both S phase and mitosis and centrosome amplification using confocal microscopy on 50 μm frozen sections. The role of estrogen and progesterone on Wnt gene expression has also been studied in both intact and PRKO mice.

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Jeffrey Rosen 9/29/97

PI - Signature Date

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INTRODUCTION

A woman's reproductive history is one of the principal determinants of her susceptibility to breast cancer. An early full-term pregnancy is protective and the length of time between menarche and the first full term-pregnancy appears to be critical for the initiation of breast cancer. This study is based upon the hypothesis that the protective effects of an early pregnancy and lactation result from estrogen (E) and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific "local mediators", i. e. growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and end bud (TEB) growth and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. No molecular markers are available to identify and follow the fate of these susceptible cells, yet this information is required to develop effective diagnostic tools and preventive therapies for breast cancer. Thus, the initial objective of this study is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. To do so, genes expressed in the TEBs of the nulliparous rat mammary gland will be isolated, cloned and characterized. These genes will then serve as molecular markers in TEB cell fate studies. Candidates for TEB molecular markers may include cell cycle factors, proteins which interact with the extracellular matrix, cytoskeletal elements and growth factor receptors. In addition, we proposed to define the topology and compare stages of the cell cycle of susceptible and refractory cells, and to identify local mediators of E- and P-treatment in the end buds and surrounding stroma, and characterize the changes in their expression patterns.

The following specific tasks were proposed for the third twelve month period of this proposal.

Task 2c. Northern analysis to follow the fate of these unique gene transcripts during mammary development and carcinogenesis.

Task 3. Definition of the topology and cell cycle analysis of susceptible and refractory cells.

Task 4. Identification of local mediators of E- and P-treatment in the end buds and surrounding stroma and characterization of the changes in their expression patterns. Specifically, the characterization of changes in the levels of expression of known growth factor family members as a function of E- and P-treatment by quantitative RT-PCR.

BODY

Materials and Methods

Animals

Wistar Furth rats with an inbred genetic background were obtained from Harlan Sprague Dawley, Inc. USA. Mouse tumors and cell lines were provided by Dr. Daniel Medina, Baylor College of Medicine. All animals were maintained and sacrificed according to IACUC approved guidelines.

Cell lines

COS 1 cells were obtained from ATCC and cultured as per their recommendations. The TM12, TM2L and TM10 cell lines were provided by Dr. Daniel Medina (Schwartz & Medina, 1987; Kittrell et al., 1992)

Probes and antibodies

A full length p190-B cDNA expression construct was a generous gift of Dr. Peter Burbelo, Laboratory of Developmental Biology, National Institute of Dental Research. The full length adrenomedullin cDNA was the kind gift of Dr. Junichiro Sakata, First Department of Internal Medicine, Miyazaki, Japan. Rabbit polyclonal antibodies for p190-B were raised against two synthetic peptides and were affinity purified. The secondary antibody and peroxidase conjugated antibodies were purchased from SantaCruz Biotechnology.

Cell culture and transfection

In order to obtain cells expressing p190-B, 5μg of plasmid DNA containing a neomycin resistance gene and 5ug of stuffer plasmid DNA were co-transfected into COS 1 cells using the lipofectamine reagent (Boehringer Mannheim). After 5-8 hrs the transfection medium was supplemented with medium containing 20% fetal bovine serum (FBS). The media was replaced after 18 hrs with fresh media containing 10% FBS. Cells were harvested after 24hrs and stored at -80°C until analyzed.

Preparation and analysis of RNA

The fourth abdominal mammary glands from virgin, pregnant and lactating rats were dissected under anaesthesia using standard surgical procedures. Tissue was snap frozen in liquid nitrogen and stored at -80°C. Total cellular RNA was prepared using 4M guanidium isothiocynate and CsCl buoyant density centrifugation (Maniatis, 1988). RNA was fractionated on a 1.2% formaldehyde agarose gel and transferred to Hybond N+ (Amersham) membrane with 10X SSC. Hybridization was performed in Hybaid oven at 65°C using procedures recommended by Amersham.

Preparation of protein extracts and Western blot analysis

Protein extracts were prepared by lysing the cells in RIPA buffer (Upstate Biotechnology), followed by precleaning and protein concentration determination (Bio-Rad 96 well plate method). For Western blot analysis, 50-100 μg of protein extract from transfected cells or tissue was subjected to electrophoresis through a 6% PAGE-SDS gel. Proteins were electroblotted to PVDF membranes. Filters were blocked with 3%NFDM in TBST and incubated for 1 hr with affinity purified polyclonal antibody for p190-B diluted (1:1000) in blocking buffer. This was followed by incubating the blot with secondary antibody (goat anti rabbit) diluted (1:2000) in blocking buffer. Detection was performed with the enhanced chemiluminescence detection system (Pierce Chemicals).

Cell kinetics

Mammary glands representing additional positive controls were excised from 104 day old virgin rats. BrdU (50 μg/kg) was injected ip at 104 days of age and animals were sacrificed at 4 hr intervals during the following 28 hr period. An additional 30 rats were subjected to double exposure to MNU at 97 and 104 days and then divided into two subgroups. Subgroup 1 received BrdU injections at 104 days and their mammary glands collected every 4 hrs for 28 hrs. Subgroup 2 received BrdU injections at 109 days and their mammary glands collected every 4 hrs for 28 hrs.

All mammary glands were frozen at -80°C, sectioned on a cryomicrotome at 50 μm thickness, and collected on washed glass slides. The sections were permeabilized by treatment with 0.5% Triton-X 100 in PBS for 4 min at RT. The tissue slices were then fixed in 4% formaldehyde in PBS for 15 min at RT. After treatment with 2.5N HCl at room temperature for 40

min to denature the DNA, the slices were stained with mouse-anti-BrdU antibodies (Boehringer Mannheim) and rabbit anti-keratin 14 (1:500) at 37°C for 3 hrs.

After washing, the samples were incubated with a mixture of three secondary antibodies (FITC-conjugated anti-mouse; a goat-anti-rabbit FITC conjugated anti-K14; and an RITC conjugated goat anti-rabbit antibody). The latter combination of antibodies at a 1:1 mixture produces yellow color in myoepithelial cells. The tissue slices were then stained with propidium iodide (1 μg/ml). This combination of treatments produces a tri-colored histological preparation that enables us to identify and analyze the proliferative compartment of the mammary gland (S-phase cells are green, all other nuclei are red and the myoepithelial cells are stained yellow). The slides were examined by a laser scanning confocal microscope (Molecular Dynamics), operating at an optical section step size of 5 μm thickness. The images are recorded as TIFF or JPG files for cell cycle scoring.

Combination BrdU/Histone H3 staining for mitotic cells

In order to determine complete cell parameters, it is necessary to score the % of BrdU-positive mitotic cells following an earlier pulse with BrdU. Until recently, this was not possible using confocal microscopy. This limitation was overcome by staining cryosections of rat mammary glands with a new mitosis-specific antibody made against the phosphorylated form of the amino terminus of histone H3 (anti-H3) obtained from Dr. C. David Allis (University of Rochester). Following the anti-BrdU step described above, the slides were washed and exposed to the anti-H3 (1:1000 in PBS plus 1% BSA for 3 hrs). After washing, the samples were treated with Texas Red-labeled goat anti-rabbit IgG. Using this scheme, BrdU-labeled nuclei are green, mitotic cells are red, and BrdU-labeled mitotic cells are yellow.

Centrosome amplification as a marker for genome instability

Centrosome replication is a useful marker for identifying cells in the cell cycle and for detecting early stages of genome instability (Fukasawa et al., 1996). We adapted this technique for analyzing the cells of the rat mammary gland as well as MNU-induced mammary tumor cells. Cryosections of mammary glands and tumors were obtained as described earlier. In addition to the other antibodies, the sections were incubated with SPJ anti-centrosome serum provided by Dr. Ron Balczon (University of South Alabama) at a dilution of 1:500 for 3 hrs at 37°C, followed by exposure to FITC-goat-anti-human IgG for 3 hrs at 37°C. Scoring was accomplished by counting the number of stained nuclei and the corresponding number of centrosomes in sections of both mammary gland and tumor.

Isolation and analysis of nuclear matrix proteins

A full set of 2-D patterns of nuclear matrix proteins from TEB, TEB-associated stroma, AB and AB-associated stroma was obtained by the following procedure. The mammary glands from 8-10 rats (45 day old) were excised after injections of 0.5% Trypan Blue into the nipple to reveal the mammary tree. Each gland was dissected into two anatomical regions consisting of the mid-gland, rich in alveolar bud (ABs) and the terminal region, rich in terminal end buds (TEBs) as shown in Figure**?.** After treatment with collagenase (2 mg/ml) at 37°C for 1.5 hrs, the tissues were washed in DMEM and centrifuged at 500Xg multiple times in order to separate stroma from glands. The process of digestion and separation was monitored by microscopy assays, assuring that clean preparations of ABs, TEBs and stroma were obtained. This procedure produced four fractions: (1) TEBs, (2) TEB-associated stroma, (3) ABs, and (4) AB-associated stroma. Four fractions were placed into CSK buffer and homogenized in 0.5% Triton X-100 to release lipids and soluble proteins. The samples were then centrifuged and incubated with 50-200 μg/ml RNase-free DNase I in CSK buffer at 32°C for 45 min, followed by extraction in 0.25 M ammonium sulfate in CSK buffer. After centrifugation, the pellet was incubated in 1.6 M NaCl for 5 min at

room temperature. The pellet was then washed in CSK buffer and frozen at -80°C. The samples were then sent to Zaxis, a biotech company in Audson, Ohio, who processed and stained the 2-D gels according to the high resolution color-based silver staining procedure.

Results and Discussion

Task 2c. Northern analysis to follow the fate of these unique gene transcripts during mammary development and carcinogenesis.

DD-PCR was used to identify candidate genes that may play a role in mammary gland development and neoplasia. As described previously, a total of 14 clones were identified. The RNA expression levels of EDD-C3, C6, C11, C14, C15, C18, G5, G6 and G7 were analyzed by RT-PCR in an attempt to identify clones which are expressed predominantly in the TEB RNA. Four clones; EDD-G5, G6, G7 and C18 were more highly expressed in the TEB RNA. Interestingly, EDD-G6, G7 and C18 are clones which were originally isolated from a DD-PCR experiment in which two separate RNA pools were used for duplicate PCR runs. These results are promising, however there were problems with variability of clone expression between the individual RNA preparations studied. This is most likely due to variation in the amount of ductal and stromal contamination in the original pooled dissection fractions used for RNA isolation.

Because RT-PCR analyses of pooled fractions does not permit a conclusive identification of the cell types expressing specific RNA transcripts, experiments are in progress to localize the expression of clones EDD-C2, C12, G5, G6, G7 and C18 by *in situ* hybridization in the virgin rat mammary gland. The major problem we have encountered is the ability to perform *in situ* hybridization for scarce mRNA transcripts at sufficient resolution to localize transcripts at the cellular level. Initially, *in situ* RT-PCR (IS-PCR) of differential display clones was attempted to localize mRNA expression to specific cell types in the virgin gland. However, there was little success in the virgin mammary gland due to high background levels, possibly due to DNA and the rTth polymerase. We are currently optimizing more conventional methods of *in situ* hybridization using ^{35}S-UTP-labeled riboprobes.

While some of the clones identified by DD-PCR may be false positives or less interesting, e.g. cytochrome C oxidase, four clones (three of unknown identity) are preferentially expressed in the TEB fraction. One of these DD-PCR cDNAs, EDD-G5, which was originally identified as an Expressed Sequence Tag (EST), has recently been found to be related to a novel mammalian LDL family member termed LR11. Additionally, both adrenomedullin, a secreted peptide factor, and p190-B, a new Rho GAP protein, initially appear to be expressed in the TEB and may play important roles in regulating TEB proliferation and apoptosis.

Of the 14 clones originally identified and sequenced, two clones 98-100% homologous with known genes, were selected for detailed analysis because of their potential importance in mammary gland development. For example, DD-PCR clone C-2 was found to be 98% homologous to p190-B. Similarly DD-PCR clone C-12 was 100% homologous to adrenomedullin. Our immediate goal, therefore, was to investigate the role these genes may play in mammary gland development and neoplasia and to determine if they are developmentally regulated. However, when these DD-PCR clones that were only 241 and 108 bp in length were employed as probes for Northern blot analyses, the signal to noise ratio was not sufficient to detect specific transcripts in total RNA preparations. Although we were previously able to detect expression of these clones in the virgin mammary gland using RT-PCR this technique was not amenable to quantitative studies in the absence of appropriate internal standards. One solution to this problem was the generation of longer cDNA probes. Accordingly, we have prepared a cDNA library from RNA isolated from the mammary glands of 45-day old rats. This library will be screened to obtain full length cDNAs encoding the previously described three novel clones isolated by DD-PCR and their complete

sequences determined and compared to known sequences in the database. We were fortunate, however, to obtain full-length clones encoding both p190-B and adrennomedulin from other investigators to use in Northern blot analysis.

p190-B is developmentally regulated

We have analysed the expression of p190-B in various adult rat tissues and in mammary glands of Wistar Furth rats at different developmental stages. RNA from p190-B transfected COS 1 cells was used as a positive control. As expected from earlier results (Burbelo et al., 1995), p190-B was found to be expressed in several adult tissues including lung, liver, kidney, brain and heart. It is expressed at much lower levels in ovary and uterus (Fig. 1). Interestingly, p190-B was not only expressed in mammary gland, but was also developmentally regulated. The highest expression was seen in virgin mammary glands of a 45 day old animal, the time at which TEB activity is maximal. The expression of p190-B mRNA was found to be slightly down regulated in the virgin mammary glands of a 120 day old animal. The mRNA level increased once again at mid-pregnancy, but was not detectable by day 18 of pregnancy. To confirm these observations, the experiment was repeated using two different concentrations of RNA (Fig. 2A) and expression quantitated using the Molecular Dynamics Phosphoimager (Fig. 2C). The ethidium bromide stained agarose gel provided a control for RNA loading (Fig. 2B), but the blot still needs to be reprobed with another epithelial marker gene, such as that encoding keratin-18 to normalize for changes in epithelial cell populations during development. Studies are in progress to determine if p190-B expression is influenced by E and P, and to compare levels of expression in the mammary glands of age-matched nulliparous and parous rats.

Based on our observation that p190-B was expressed at higher levels in the mammary glands at stages representing increased proliferation, i.e. 45-day virgin and early-mid-pregnancy, and was down regulated at late pregnancy, we have also performed a preliminary analysis of its expression in a limited number of established mouse mammary cell outgrowth lines with differing tumorigenicities. The expression of p190-B mRNA appears to be increased in the more highly tumorigenic cell lines and tumors (Fig. 3A & B). The expression of p190-B mRNA was more abundant in high grade tumors as compared to more differentiated cell types (TM 2L) (Fig. 3). These preliminary studies will be extended to examine p190-B expression in a series of hormone-dependent MNU-induced rat mammary gland carcinomas following castration and E and P readministration.

Detection of p190-B in mammary gland extracts

In order to detect p190-B expression in the mammary gland extracts of Wistar Furth rats by Western blotting, we raised antisera against two different peptides from the carboxyterminus (a.a. regions 1018 - 1033, peptide-1 and a.a 1130 - 1145, peptide-2) of human p190-B. This region is absolutely conserved between rats and human. The antibodies were affinity purified and titrated by ELISA. Of the two antibodies, the rabbit polyclonal antibody against peptide-2 was of higher affinity. We have thus used only this antibody for our further studies. The specificity of the antibody was determined by assaying p190-B expression in COS 1 cells transiently transfected with a p190-B expression vector. This provided the positive control necessary to characterize these affinity purified rabbit polyclonal antipeptide antibodies. The peptide -2 antibody recognizes the expected 190 kDa protein with an additional 66 kDa crossreactive protein (Fig. 4A). The expression of the p190-B protein was also detected in nontransfected cells, although at a decreased level, possibly because COS 1 cells are derived from kidney cells of African green monkey, a tissue known to overexpress p190-B. The specificity of the antibody was demonstrated by competition by peptide-2 (Fig. 4B), but not by peptide-1 (Fig. 4C). We also detected p190-B expression in extracts of the mammary gland of a rat at day 6 of pregnancy, and of the kidney (data not shown). The protein is expressed in both tissues, although in much lower levels than the transfected COS 1 cells. This antibody will now be employed to study the expression and

localization of p190-B during mammary gland development using both conventional immunohistochemistry as well as for confocal microscopic analysis on thick frozen sections as described below.

Expression of adrenomedullin mRNA in the mammary gland

EDD-C12 is rat adrenomedullin, a secreted peptide factor that is expressed in a variety of tissues (lung, heart, kidney, brain and mammary gland) and a potent vasodilator. Polyclonal antiserum and peptide antigen to adrenomedullin have been obtained from Dr. Frank Cuttitta at the NIH. The rabbit polyclonal antiserum was generated against the most C-terminal 30 amino acids of the adrenomedullin active peptide (116-146 a.a.). This antiserum reacts with the 18 kDa proprotein, a 14 kDa intermediate form and the 6 kDa active peptide. The polyclonal antiserum has been used to determine the temporal and spatial expression of adrenomedullin in the mammary gland by immunohistochemistry. As reported previously in the year 2 progress report, immunohistochemistry was performed with fixed sections of rat mammary glands from various time points throughout mammary development. Adrenomedullin was localized to the cytoplasm of epithelial and stromal cells of the virgin gland. Staining was most prominent in TEB and ductal epithelium. However, staining was also detected in the alveolar buds, stroma, blood vessels and lymph node. This adrenomedullin staining could be competed with the adrenomedullin peptide but not with a nonspecific peptide. In the 12 day pregnant gland, adrenomedullin expression was pronounced throughout the cytoplasm of alveoli, ducts and stroma. Interestingly, in the 18 day pregnant gland adrenomedullin was localized to the nucleus and cytoplasm of alveoli and cells of the stroma. Nuclear staining was also observed in lactating (2 days) and involuting (3 days) tissue but to a lesser extent. The cytoplasmic staining of the epithelium and stroma also continued through lactation and involution. In all cases, adrenomedullin staining could be competed with the specific peptide.

Because adrenomedullin is a secreted growth factor that can act in both a paracrine and autocrine manner we wanted to complement these studies of immunolocalization to determine the levels of adrenomedullin mRNA in the mammary gland at different stages of development. Once again, we were fortunate to obtain a full length cDNA probe from Dr. J. Sakata. As reported previously, adrenomedullin mRNA was found to be universally expressed with kidney, heart and ovary showing the highest expression (Fig. 5). Moderate expression was seen in brain and virgin mammary gland, and uterus, lung and liver show low levels of expression The mRNA appears to be slightly down-regulated during lactation. In order to determine which cell types are synthesizing adrenomedullin mRNA during mammary gland development it will be necessary to complement these studies with *in situ* hybridization analysis. To accomplish this goal the full-length cDNA will be cloned into a vector to generate both sense and antisense ^{35}S-riboprobes for *in situ* hybridization.

Task 4. Identification of local mediators of E- and P-treatment in the end buds and surrounding stroma and characterization of the changes in their expression patterns. Specifically, the characterization of changes in the levels of expression of known growth factor family members as a function of E- and P-treatment by quantitative RT-PCR.

E and P regulation of local growth factors

The regulation of the Wnt and Fgf growth factor families by steroid hormones appears to play an important role in mammary tumorigenesis and breast cancer. We have studied steroid hormone regulation of Wnt gene expression in intact and progesterone-receptor knockout (PRKO) mice. Mouse mammary gland development has also been examined in PRKO mice using reciprocal transplantation experiments to investigate the effects of the stromal and epithelial PRs on

ductal and lobuloalveolar development (Humphreys et al., 1997, copies appended). The absence of PR in transplanted donor epithelium, but not in recipient stroma, prevented normal lobuloalveolar development in response to estrogen and progesterone treatment. Conversely, the presence of PR in the transplanted donor epithelium, but not in the recipient stroma, revealed that PR in the stroma may be necessary for ductal development. Stimulation of ductal development by the progesterone receptor may, therefore, be mediated by a unknown secondary signaling molecule, possibly a growth factor. The continued stimulation of the stromal PR appears to be dependent on reciprocal signal(s) from the epithelium, and in the PRKO mouse this feedback loop is interrupted. Thus, the combination of gene knockout and reciprocal transplantation techologies has provided some new insights into the role of stromal-epithelial interactions and steroid hormones in mammary gland development. Wnt-2 gene expression is maximal in the mammary stroma of 4-6 week old mice and is repressed by estrogen, while Wnt-5b is expressed in lobuloalveolar cells and is dependent upon the presence of intact PR in the epithelium.

Task 3a. Definition of topology and cell cycle analysis of susceptible and refractory cells by confocal microscopy (continued from year 2).

As reported in the Progress Report for year 2, our experiments have continued to focus on determining the growth fraction (GF), labeling index and fraction of cells in S-phase in control 45 and 96 day old rats from thick frozen sections examined in 3-D by confocal microscopy. The control data-base has now been extended by analyzing cell kinetics in the mammary glands from 15 virgin rats injected with BrdU (50 μg/kg) at 104 days, after which two animals were sacrificed every four hours for 24 hours. The results to date are consistent with published accounts of cell kinetics in older virgin rats. In another group, MNU was administered to virgin animals at days 97 and 104 days. Subsequently, these animals were divided into two groups: group one consisted of 15 animals that received BrdU immediately after the second exposure to MNU at 104 days, group two consisted of 15 animals that received BrdU injections five days following the last exposure to MNU. Two animals were sacrificed every 4 hrs following each of these treatments and the mammary glands were excised, frozen at -80°C and later fixed and cryosectioned for confocal microscopic analysis as described in the 02 year report. At the time of this report, cell cycle analysis by confocal microscopy is being conducted on tissues from the 30 animals in this experiment. The data is being collected and will be compared to the 45, 96 and 104 day old control animals.

Our efforts to completely analyze the cell cycle in rat mammary glands have been hampered by our inability to accurately determine the % of BrdU-labeled mitotic cells in thick cryosections. This step is essential for establishing the duration of M and G_2 phases. We have recently overcome this problem through the availability of a new antibody specific for the phosphorylated form of the amino terminus of histone H3 (ser10), a specific marker for condensed chromatin in mitotic cells (Hendzel et al, in press) as shown in Figure 6. We have now added this probe to our standard protocol, making it possible to determine the complete cell cycle along with labeling index and growth fraction from thick frozen sections.

We have also adapted a new procedure for identifying mammary gland cells that display genome instability, an early marker for progression to malignancy. Our test is based on a recent report that genomic instability in vitro and in vivo is signaled by centrosome amplification (Fukasawa et al., 1996; Wang et al., 1997). In normal tissue cells, the centrosome, a discrete cytoplasmic domain that contains centrioles and forms the spindle poles in M, replicates once and only once in each cell cycle. Thus, a cell in G_1 contains a single centrosome, while cells in late S-, G_2- and M-phase contain a pair of centrosomes. When cells become transformed, centrosomes depart from this duplication cycle and become amplified resulting in multiple (greater than two) centrosomes in each cell. Using an anti-centrosome antibody (Balczon et al, 1995), we analyzed centrosome numbers in TEB and stroma cells as well as MNU-induced rat mammary gland tumors;

and as shown in Figure 7, centrosome number was found to be normal in TEBs and stromal cells but highly amplified in tumor cells. In order to make this procedure work on mammary gland cells *in vivo*, we adapted the centrosome antibody technique, formerly used only in tissue culture, to thick (5 μm) frozen sections of mammary glands and tumor tissue (He and Brinkley, 1997, manuscript in preparation). This procedure offers an promising new strategy for detecting early progression to malignancy in mammary glands and will be adapted for analyzing susceptible and refractory cells in mammary glands.

Task 1b and c. Isolation and analysis of nuclear matrix proteins in normal mammary gland and mammary tumors by 2-D PAGE (continued from year 2).

One of our aims has been to analyze the nuclear matrix proteins components to terminal end buds (TEBs) and alveolar buds (ABs) in control rat mammary glands and in glands from animals treated with E and P hormones and MNU. As described in a previous progress report, we have succeeded in carrying out a comparative analysis of nuclear matrix proteins from the TEBs and ABs and adjacent stroma. Forty-five day old rats were injected with 0.5% Trypan Blue in the nipple revealing the mammary gland tree. Regions such as those shown in Figure 8 were separately collected under the dissecting microscope by digesting with collegenase (2 mg/ml) until ducts and TEBs free of stroma were free of surrounding stroma. The stroma cell fraction collected from either the TEB area or the AB area was separated by centrifugation as shown in a previous progress report. Our progress in obtaining gels from these samples has been delayed somewhat due to personnel changes in the Protein Analysis Core Laboratory at Baylor. When we failed to obtain satisfactory results with the new personnel, we were forced to contract with a private company (see Methods Section) to provide the 2-D silver-stained gels. Results shown in Figure 9 indicate that a specific nuclear matrix protein profile was found to be specifically localized in either TEB or AB, as reported in the previous progress report. A somewhat different profile was found in the stroma preparations. These gels indicate that specific marker proteins can be found in the four compartments sampled and that stroma associated with the TEB region may express proteins different from the stroma collected from the AB region. We have continued to analyze nuclear matrix proteins in these regions in additional samples. In addition, nuclear matrix proteins were analyzed in two separate samples of MNU-induced rat mammary tumors and compared to patterns found in both mammary epithelial and stromal cells. As shown in Figure 9, at least two peptides were found to be specifically expressed in both tumors that were not found in the normal differentiated tissue samples. Some gel spots, possibly representing keratins (acidic peptides of approximately 55kD), indicate that both mammary tumors were derived from epithelial cells of the TEB.

CONCLUSIONS

Thus far, 14 clones isolated from the TEB DD-PCR fraction have been sequenced. Three clones of unknown identity are preferentially expressed in the TEB fraction. A cDNA library prepared from RNA isolated from 45 day old rats is currently being screened for full length clones. Full length cDNA clones to two known cDNAs encoding p190-B and adrenomedullin have been obtained and used for Northern blots to measure the levels of their respective mRNAs during mammary gland development. Antibodies to p190-B and adrenomedullin are being used to localize and study their function. An affinity purified antipeptide antibody to p190-B has been demonstrated to recognize authentic p190-B expressed in COS-1 cells. Both of these proteins appear to be expressed in the TEB and may play important roles in regulating TEB proliferation and apoptosis. Procedures have been developed to isolate nuclear matrix proteins from the TEB and preliminary 2D PAGE analysis has identified several unique proteins in this fraction. Procedures have also been optimized for measuring cell cycle kinetics and centrosome amplification in frozen sections using confocal microscropy. Finally, the steroid hormone regulation of Wnt-2 and Wnt-5B have been studied in intact mice and PRKO mice. Estrogen exerts an acute inhibition of stromal Wnt-2 expression in the absence of the progesterone receptor,

while estrogen and progesterone are required for the chronic induction of Wnt-5b in lobuloalveolar cells. Overall, the progress made during this past year is consistent with the original tasks proposed for the third year in the Statement of Work.

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FIGURE LEGENDS

Figure 1: Detection of p190-B expression in various adult rat tissues of a 120 day old virgin rat with a full length cDNA probe. Almost all of the tissues express a 6.4 kb long transcript. Total cellular RNA from non-transfected (NT) and p190-B transfected COS-1 cells (T) was used as a positive control (lanes 9 and 10 shown in a separate panel on the right). Please note that the signal depicted for lanes 9 and 10 was obtained from a 4 hr exposure as opposed to overnight exposure for the remaining tissues. The lower panel represents EtBr staining of the above RNA samples. MG: Mammary gland RNA from 120 day old virgin rat.

Figure 2: A: p190-B expression is regulated during mammary development. a) Total cellular RNA (15 or 25 μg) from the 4th abdominal mammary glands of 45 day virgin (lanes 1 & 2), 120 day virgin (lanes 3 & 4), 12 day pregnant (lanes 5 & 6), 22 ug of 18 day pregnant (lane 7) and 10 day lactating (lanes 8 & 9) were probed with p190-B cDNA to detect a 6.4 Kb

transcript. Please note that p190-B is not detectable in mammary gland RNA of 18 day pregnant and 10 day lactating rats (lanes 7, 8 & 9). b). EtBr staining of above RNA samples. **B**: Phosphorimager quantitation of p190-B expression during different stages of mammary gland development. The above results were quantitated using the software Image-quant (Molecular Dynamics). The data are plotted in arbitrary relative volume units/μg RNA.

Figure 3: Detection of p190-B expression in mouse mammary tumors and cell lines. a) Total cellular RNA (20μg) isolated from DIM tumors (lanes 1 &2) and the TM series of cell lines (lanes 3,4 & 5) were analysed for p190-B expression using Northern blotting. Total cellular RNA from a 120 day old virgin rat mammary gland was used for comparison (lane 6). Note that DIM tumors and TM 10 cell lines (lanes 1,2 & 5) that are highly tumorigenic exhibit the highest expression of p190-B as compared to TM 2L (lane 4) which is more differentiated and has the lowest tumor forming potential. b) EtBr staining of above RNA samples. c) Phosphorimager quantitation of p190-B expression in DIM and TM mouse mammary tumors. The above data were quantitated for p190-B expression using the software Image-quant (Molecular Dynamics). The results are plotted in arbitrary relative volume units.

Figure 4: Western blotting with p190-B antisera. Lysates from nontransfected (NT) and transfected (T) COS-1 cells were resolved on 6% SDS-PAGE and blotted onto PVDF membranes as described in Materials and Methods. Filters were cut into strips and either A) incubated with antibody to p190-B B). incubated with Ab to p190-B that was competed with its specific peptide prior to incubation or C). incubated with Ab to p190-B that was competed with a non-specific peptide.

Figure 5: Expression of adrenomedullin mRNA (AM) in adult rat tissues and during mammary gland development. Total cellular RNA (20 μg) isolated from adult rat tissues and mammary gland at different developmental stages was probed with a rat cDNA for AM. The highest expression was seen in kidney, ovary, heart, brain and uterus (lanes 3,4,5,6 & 7). In mammary gland, expression is highest in RNA extracted from the 45 day virgin gland and at day 12 of pregnancy (lanes 8, 9 & 10). AM expression appears to be downregulated during lactation (lanes 11 & 12).

Figure 6. New procedure for cell cycle analysis. TEB from a 104 day old virgin rat that received BrdU (50 μg/kg) and sacrificed four hours later and stained with an antibody against the amino terminus of histone H3 specific for mitotic cells. BrdU positive nuclei are green, mitotic cells are shown in red, and mitotic cells that were previously exposed to BrdU are shown in yellow. All nuclei are stained with DAPI (blue). Such preparations are used to plot the % labeled (BrdU) mitosis essential for determining all phases of the cell cycle.

Figure 7. Centrosomes as markers for genome instability and malignancy. Examination of stroma cells show predominantly single centrosomes as expected since these cells are in G_0. Examination of TEB cells (middle photo at top) shows most cells contain one or two centrosomes as expected of proliferating cells. The bright green cells are stained with keratin antibodies to depict the myoepithelial cell layer. Anti-centrosome staining of MNU-induced rat mammary tumor cells (upper right) show many cells with more than the expected one or two centrosomes (green spots). The frequencies of centrosome distribution in the three samples are plotted below.

Figure 8. Mammary gland tree contrasted by injection of Trypan Blue into the nipple. Preparations such as this enabled us to dissect the mammary gland to produce a TEB-rich fraction and a AB-rich fraction as depicted as outlined by the red and blue marker.

Figure 9. Nuclear matrix proteins specific mammary gland compartments. 2-D gels of nuclear matrix proteins isolated from different compartments of 45 day old rats include TEBs, ABs, stroma from TEB, and stroma from AB region. In addition, gels from two separate tumor samples display protein spots that corresponded to spots on gels representing the TEB sample indicating likely origin of these two tumors. Such gels will be useful in determining the tissue cells from which the tumor originated.

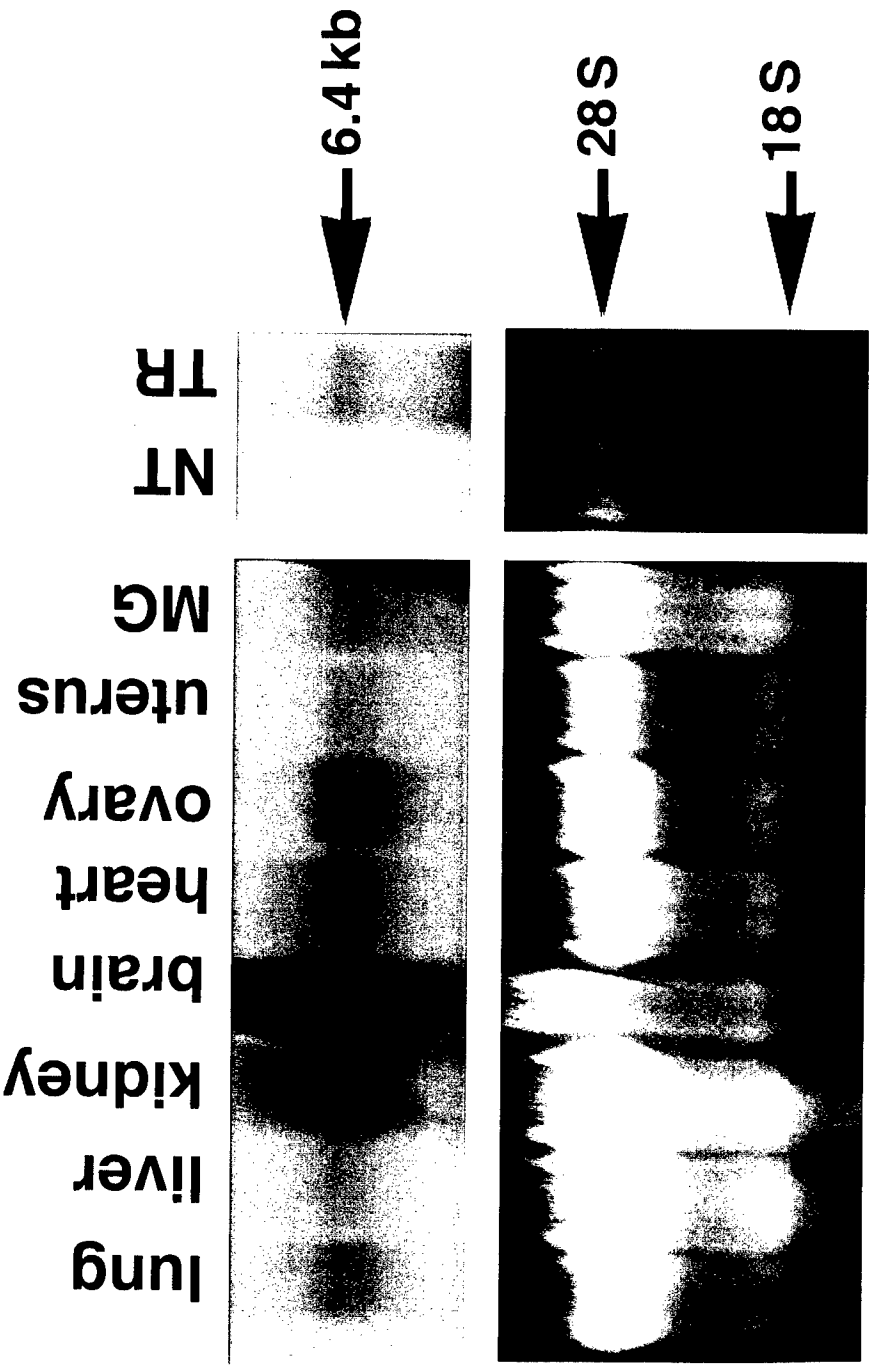
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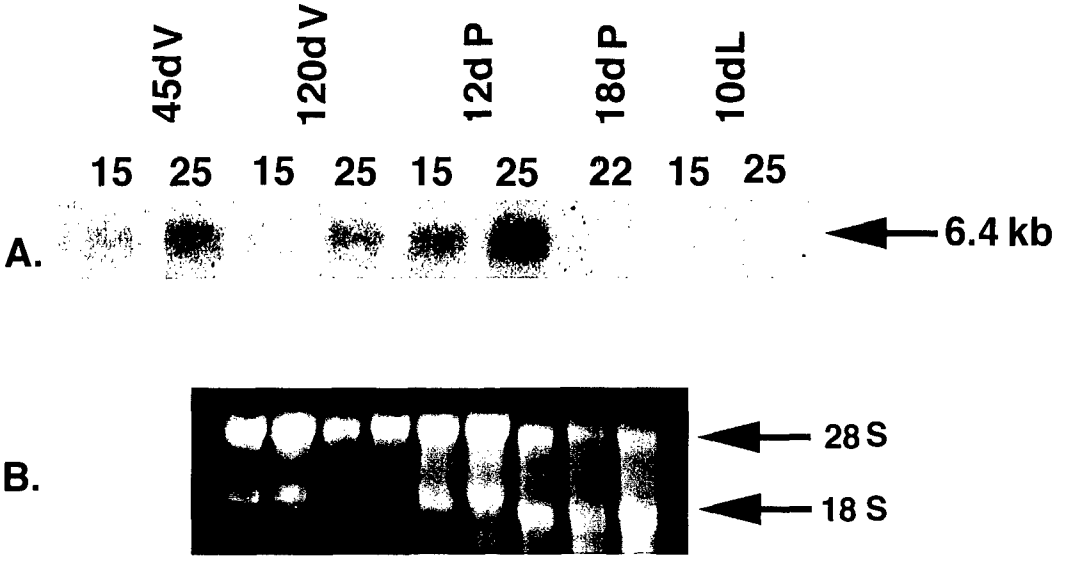
Figure 1



C. p190-B expression during mammary gland development

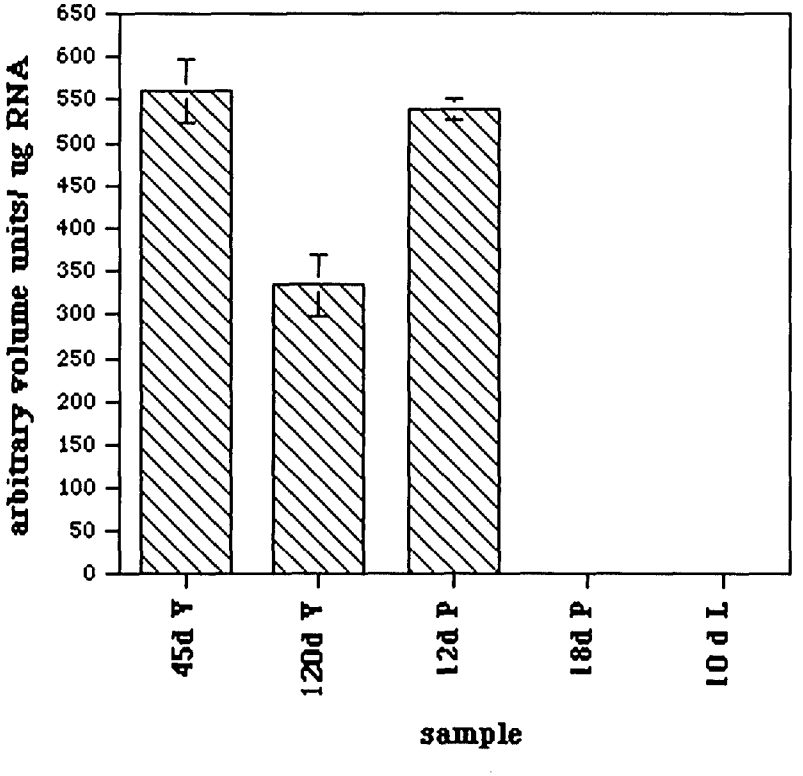
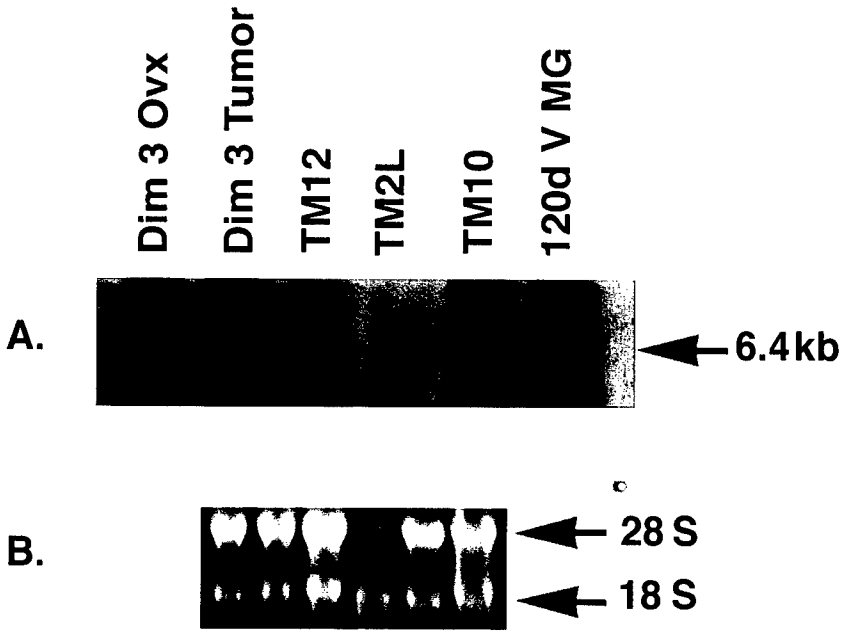


Figure 2


Dim 3 Ovx
Dim 3 Tumor
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B.
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C.
Expression of p190-B in mouse mammary tumours
arbitrary volume units
35000
30000
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Figure 3

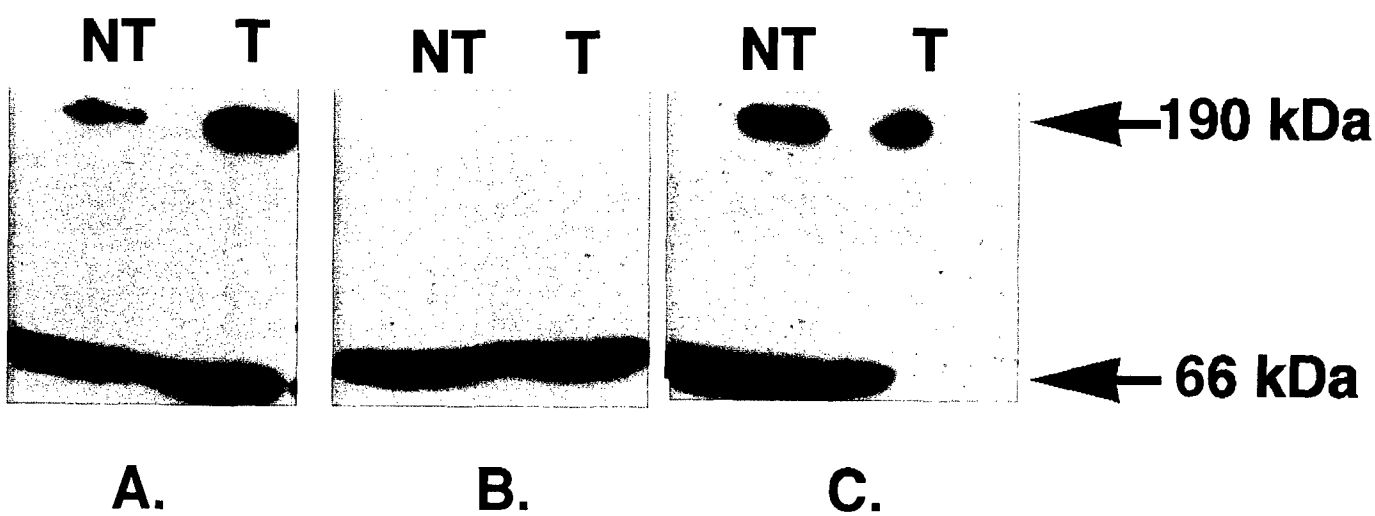

Figure 4

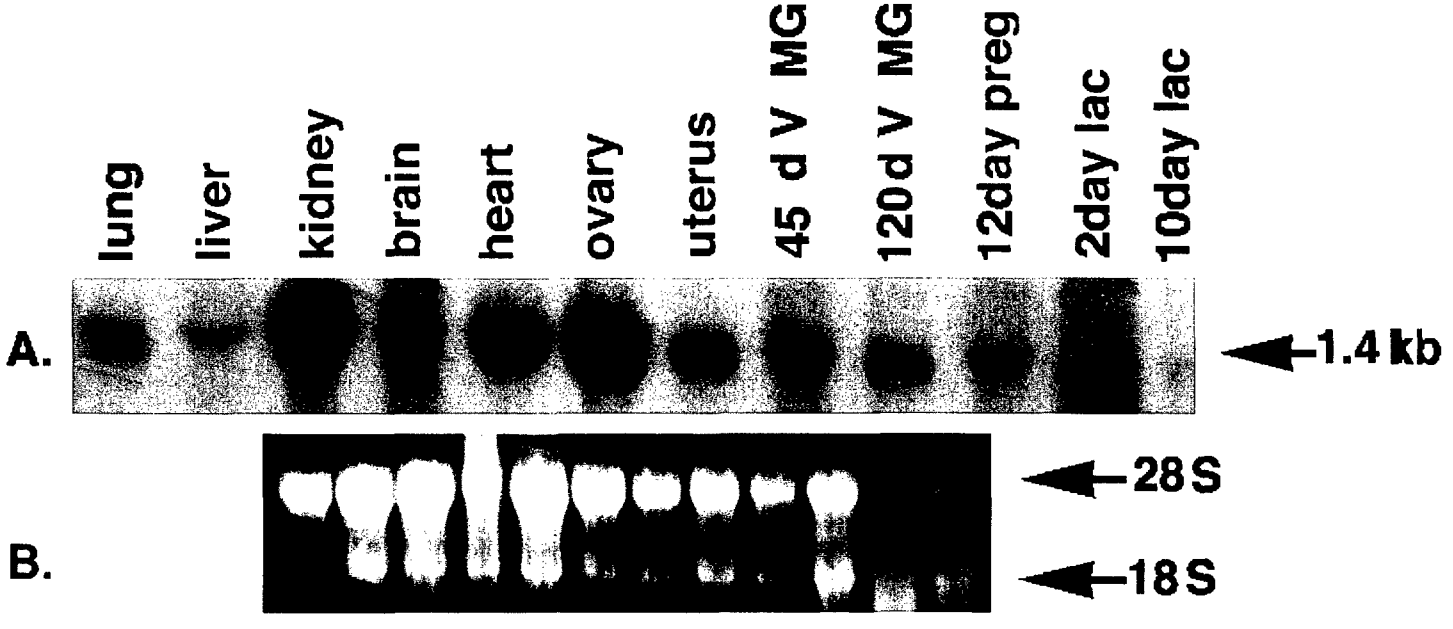
lung
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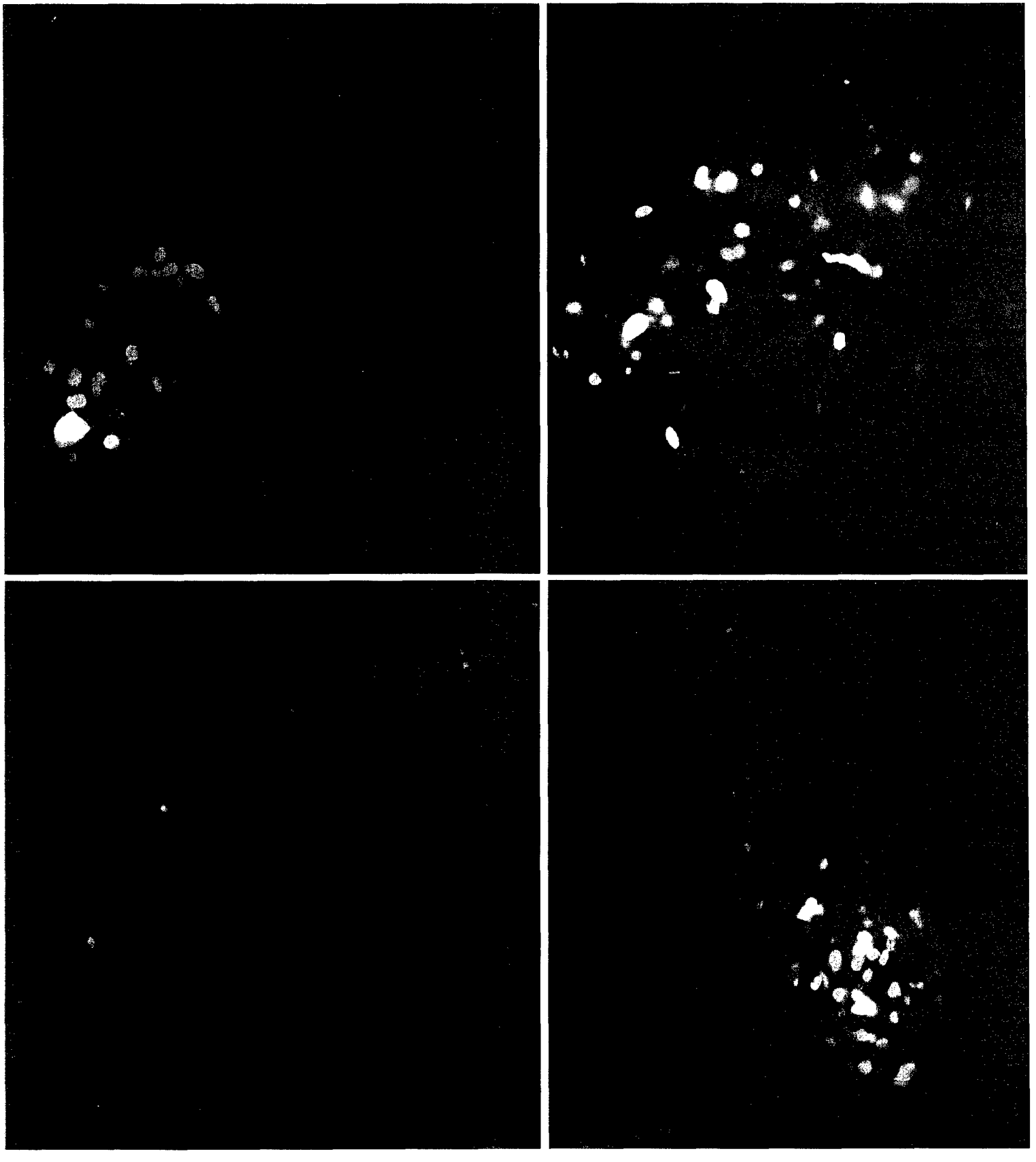
Figure 5



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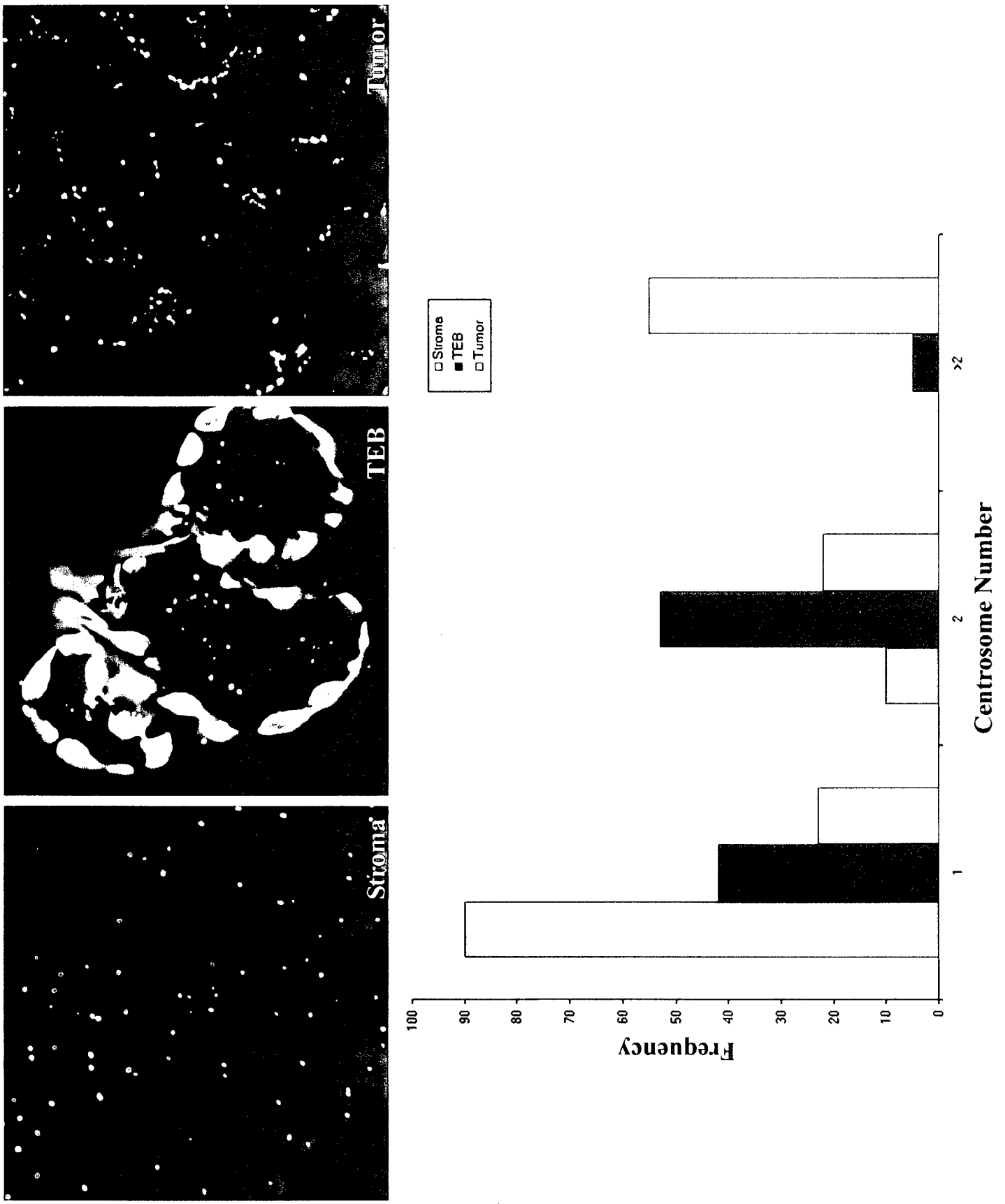
Stroma
TEB
Tumor
Stroma
TEB
Tumor
Frequency
100
90
80
70
60
50
40
30
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1
2
>2
Centrosome Number

Figure 7

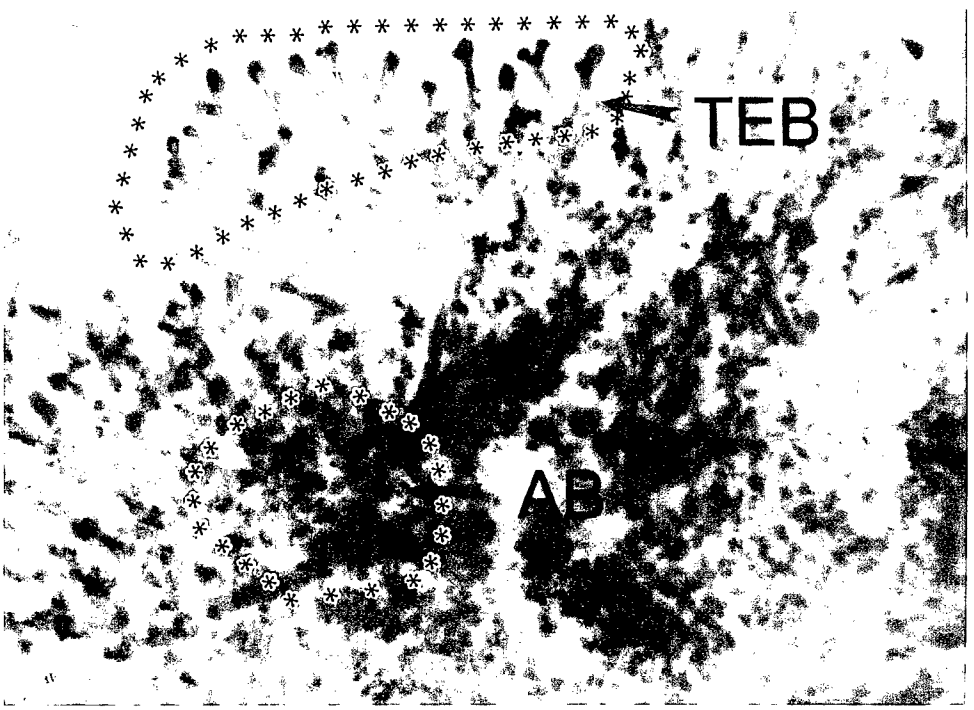

Figure 8

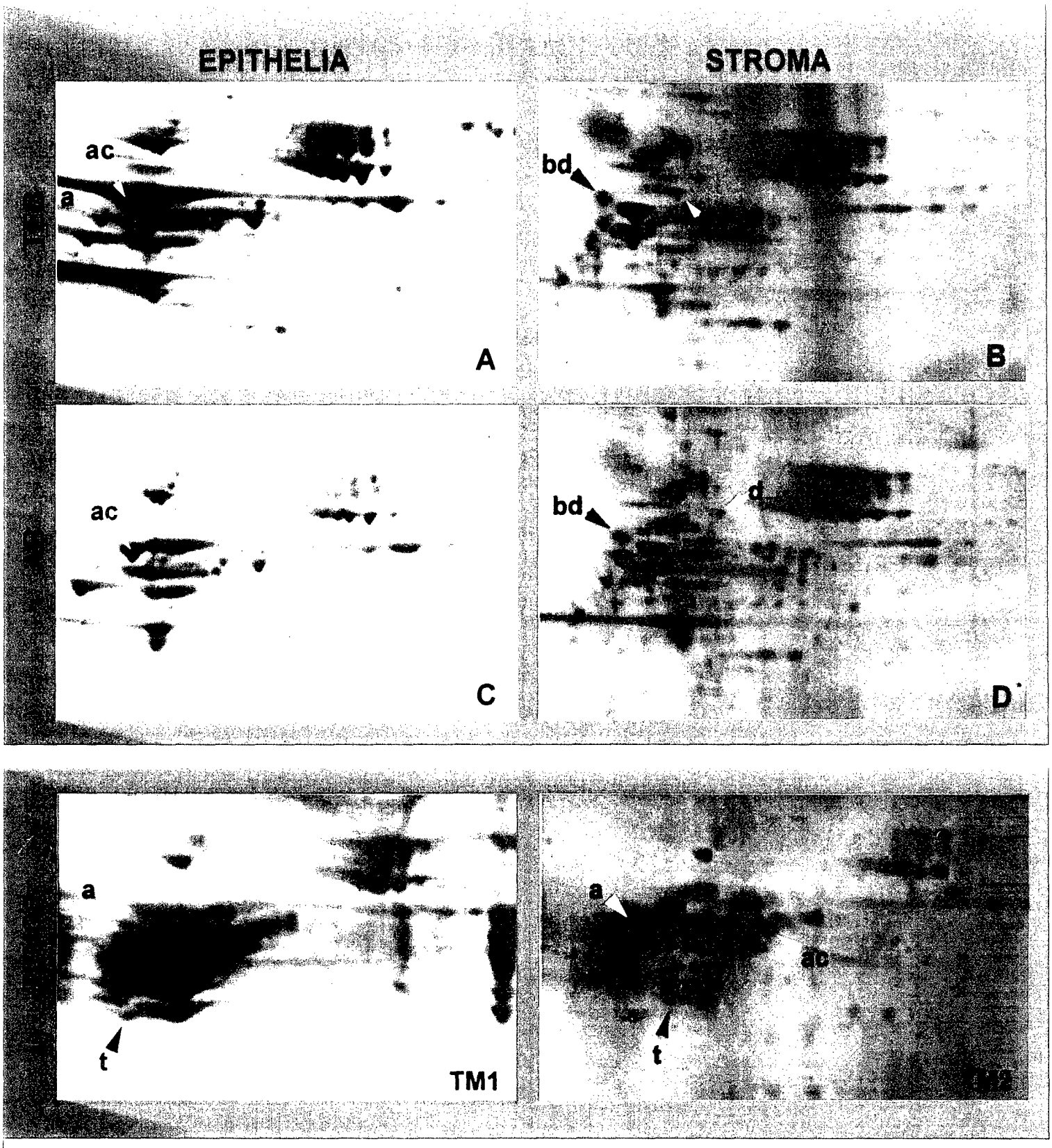

Figure 9

APPENDIX

Humphreys, R.C., Lydon, J., O'Malley, B.W. and Rosen, J.M. (1997) A role for epithelial and stromal progesterone receptors in the regulation of Wnt gene expression during mammary gland development. Mol. Endocrinol. 11:801-811.

Mammary Gland Development Is Mediated by Both Stromal and Epithelial Progesterone Receptors

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A combination of a knockout mouse model, tissue transplantation, and gene expression analysis has been used to investigate the role of steroid hormones in mammary gland development. Mouse mammary gland development was examined in progesterone receptor knockout (PRKO) mice using reciprocal transplantation experiments to investigate the effects of the stromal and epithelial PRs on ductal and lobuloalveolar development. The absence of PR in transplanted donor epithelium, but not in recipient stroma, prevented normal lobuloalveolar development in response to estrogen (E) and progesterone (P) treatment. Conversely, the presence of PR in the transplanted donor epithelium, but not in the recipient stroma, revealed that PR in the stroma may be necessary for ductal development. Members of the Wnt growth factor family, Wnt-2 and Wnt-5B, were employed as molecular markers of steroid hormone action in the mammary gland stroma and epithelium, respectively, to investigate the systemic effects of E and P. Hormonal treatment of intact, ovariectomized, and $PR^{-/-}$ mice and mice after transplantation of $PR^{-/-}$ epithelium into wild type ($PR^{+/+}$) stroma demonstrated that these two locally acting growth factors are regulated by independent mechanisms. Wnt-2 is acutely repressed by E alone, while Wnt-5B gene expression is induced only after chronic treatment with both E and P. Wnt 5B appears to be one of the few molecular markers of P action in the mammary epithelium. This study suggests that the regulation of mammary gland development by steroid hormones is mediated by distinct effects of the stromal and epithelial PR and differential growth factor expression. (Molecular Endocrinology 11: 801–811, 1997)

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Molecular Endocrinology

INTRODUCTION

Aberrant regulation of normal developmental pathways plays an important role in initiating and supporting mammary gland transformation. Both hormonal and developmental status are known to be important factors in the etiology of breast cancer. These hormonal and developmental cues are often mediated at the molecular level by a combination of systemic hormones and locally acting growth factors. Synergism among locally acting growth factors enhances and augments the diversity of potential signals transmitted to the epithelial and stromal components of the gland. This synergism can be stimulatory or inhibitory and can affect gene expression in the epithelium and mesenchyme.

Estrogen (E) and progesterone (P), in cooperation with pituitary hormones, are the primary systemic hormones required for the induction of proliferation and differentiation of epithelial and stromal cells leading ultimately to the formation of ductal and alveolar structures during mammary gland development. The interaction of E and P with GH, PRL, and insulin in regulating this differentiative process has been well documented (1). Steroid hormones also regulate the expression of a number of different locally acting growth factors, including members of the epidermal growth factor, insulin-like growth factor, and fibroblast growth factor (FGF) families (2, 3). Most of these growth factors exhibit localized effects due to protein stability, adhesion and residence in extracellular matrix, transport and secretion, and availability of receptor molecules. For this reason they are believed to act as local mediators of the differentiative and proliferative signals of the systemic hormones. Systemic regulation of locally acting growth factor activity allows for fine regulation of large-scale developmentally associated proliferative and differentiative functions.

Mammary gland development is dependent on physical, molecular, and often reciprocal, interactions between the stromal and epithelial compartments (4). The ability to recapitulate fully differentiated structures from a fragment of syngeneic parenchyma, and to

separate and recombine epithelial and stromal compartments *in vivo*, makes the mammary gland an excellent model system in which to study these interactions. Evidence for this reciprocal dependence has been demonstrated in classic recombination experiments between the epithelial and stromal androgen receptor pathways (4). The specific role of the epithelial and stromal PR in the development and differentiation of the mammary gland is unclear (5, 6).

Wnt-1, the progenitor of a family of related growth factors, was discovered in mouse mammary tumors as a result of proviral activation (7). Members of the Wnt gene family are expressed in invertebrates and vertebrates where they regulate cell fate and pattern formation (8). Wnt genes, other than Wnt-1, are expressed in the mammary glands of mice in a developmentally specific pattern (9–11). The function of these endogenous Wnt genes during mammary gland development is unknown. From these studies it is apparent that Wnt gene expression is tightly regulated and is dependent on the developmental state of the mammary gland. In BALB/c mice, Wnt-2 is expressed primarily during early ductal development, 5–8 weeks postnatally, coincident with time of PR induction by E, and is markedly down-regulated at the onset of pregnancy. Conversely, Wnt-5B transcripts are detectable in the late virgin gland at 6–12 weeks of age but increase markedly during pregnancy, reaching a peak at day 18. Wnt-5B expression is localized primarily in the ductal and lobuloalveolar cells, while Wnt-2 expression is detected in the stroma (9, 11). These results suggest that E and P may play a role in regulating Wnt-2 and Wnt-5B gene expression in both the stroma and epithelium. This restricted pattern of gene expression is indicative of molecules that may be involved in the developmental processes of the gland.

In this study the progesterone receptor knockout (PRKO) mouse (12) has been used for reciprocal transplantation experiments in syngeneic mice to investigate the distinct roles of the stromal and epithelial PR in mammary ductal and alveolar development. Wnt-2 and -5B provided specific molecular markers of steroid hormone action in the mammary gland stroma and epithelium, respectively. The PRKO mouse permitted definition of the unique effects of P distinct from those mediated by E on Wnt gene expression. This experimental approach should facilitate the identification of other steroid-mediated local growth factors on mammary gland development.

RESULTS

Epithelial and Stromal PRs Have Separate Roles in Mammary Gland Development

The mammary gland has the unique ability to recapitulate the complete ductal and alveolar structures from a transplanted fragment of syngeneic mammary epithelium (13). This characteristic allows the analysis of interactions between epithelium and stroma *in vivo*. The PR is present in both epithelial and stromal compartments of the murine mammary gland (5, 14). To establish the role of the PR in each of these compartments, reciprocal transplantation experiments were performed using epithelium and stroma derived from syngeneic 129SvEv $PR^{-/-}$ and $PR^{+/+}$ mice, respectively. $PR^{-/-}$ epithelium transplanted into the cleared fat pads of $PR^{+/+}$ mice penetrated and filled the stroma with ductal structures (Fig. 1A). Interestingly, the $PR^{-/-}$ epithelium failed to develop alveoli and to display an increase in the number of secondary ductal branches in response to steroid hormone treatment (Fig. 1B). The control ipsilateral glands from the host animal responded as expected to steroid hormone treatment with alveolar proliferation (Fig. 1, D *vs.* C). This result demonstrates that the PR in the epithelium is required for normal lobuloalveolar formation and differentiation of the epithelium. In addition, the presence of PR-regulated signaling pathway in the stroma cannot compensate for the lack of PR in the epithelium. In contrast, $PR^{+/+}$ epithelium transplanted into the cleared fat pad of $PR^{-/-}$ hosts and treated with E and P exhibited lobuloalveolar development (Fig. 2D). An increase in secondary branching in these E- and P-treated transplants can be clearly seen under higher magnification (Fig. 2F, *arrow*). However, an unexpected, marked reduction in the extent of ductal outgrowth was observed in these transplants after

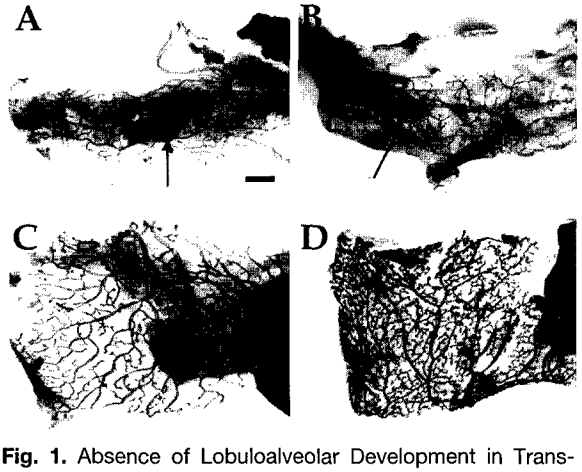

Fig. 1. Absence of Lobuloalveolar Development in Transplanted $PR^{-/-}$ Epithelium

$PR^{-/-}$ epithelium was transplanted into cleared fat pads of $PR^{+/+}$ 129SvEv mice. After 10 weeks of growth, the mice were injected subcutaneously daily with E and P, and the mammary glands were collected at day 0 (A and C) and day 8 (B and D). The *arrows* in panels A and B denote the site of transplantation. Note that in panel A the fat pad has been penetrated with ductal epithelium after 10 weeks of growth *in vivo*. Also note the increase in alveolar development in the ipsilateral $PR^{+/+}$ glands (C and D) after hormonal stimulation (compare panels C and D). *Bar* = 1.4 mm.

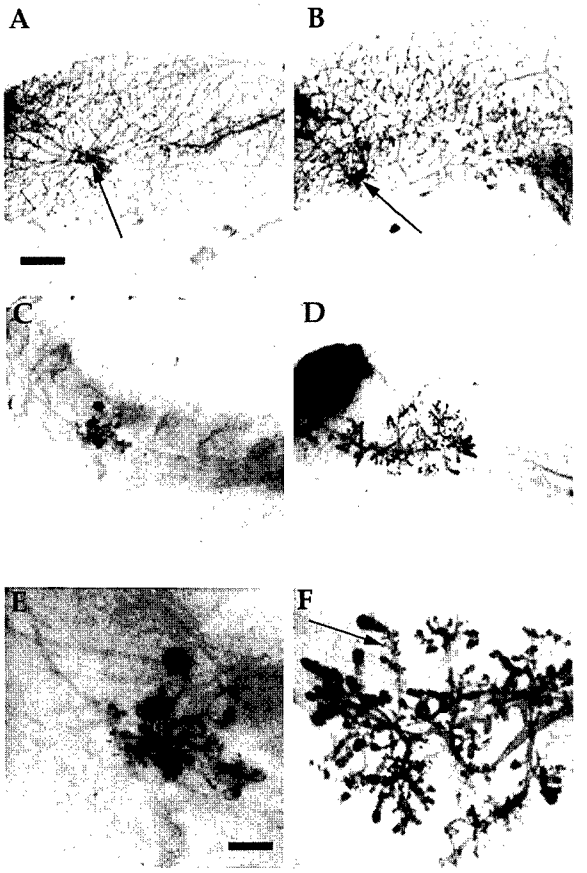

Fig. 2. The Morphological Response of $PR^{+/+}$ Epithelium Transplanted into $PR^{-/-}$ and $PR^{+/+}$ Stroma to Steroid Hormone Treatment

$PR^{+/+}$ epithelium was transplanted into $PR^{+/+}$ (A and B) and $PR^{-/-}$ (C, D, E, and F) stroma. After 10 weeks of growth, the mice were injected daily with E and P subcutaneously, and the mammary glands were collected at day 0 (A, C, and E) and day 8 (B, D, and F). Alveolar formation is evident in $PR^{+/+}$ (epithelium) $PR^{-/-}$ (stroma) after 8 days of E and P treatment (*arrow* in F). Note the reduction in ductal development in $PR^{-/-}/PR^{+/+}$ (D) compared with both $PR^{+/+}$ (B) and $PR^{-/-}$ epithelium (Fig. 1, A and B). $PR^{+/+}$ epithelium in $PR^{+/+}$ stroma responds to steroid hormone treatment with extensive alveolar growth and an increase in secondary branching (B). The *arrows* in A and B define the site of transplantation. Magnification in panels A, B, C, and D is defined by *bar* in A = 2 mm. Magnification in panels E and F is defined by *bar* in E = 0.75 mm. ($PR^{-/-}/PR^{+/+}$, n = 4 and $PR^{+/+}/PR^{+/+}$, n = 4).

10 weeks of growth (Fig. 2C), as compared with the $PR^{+/+}$ (Fig. 2, A and B) and the $PR^{-/-}$ (Fig. 1A) epithelium transplanted into the $PR^{+/+}$ stroma. The same $PR^{+/+}$ epithelium transplanted in $PR^{+/+}$ stroma responded as expected to steroid hormone treatment with extensive alveolar growth and an increase in secondary branching (Fig. 2B). The outgrowths from the $PR^{+/+}$ epithelium transplanted into the $PR^{-/-}$ stroma also displayed unusual terminal endbuds (Fig. 2, C and E).

E and P Treatment Represses Wnt-2 Gene Expression in the Mammary Glands of Ovariectomized (ovx) and Intact Mice

The role of E and P in regulating Wnt gene expression has been implied from the pattern of Wnt gene expression observed in normal mammary gland development (9–11). In particular, Wnt-2 and Wnt-5B display dramatic and inverse changes in gene expression levels at the onset of pregnancy. Wnt-2 appears to be expressed primarily in the mammary gland stroma, and Wnt-2 transcripts have been detected in the cleared mammary fat pad (9, 11), whereas Wnt-5B is expressed specifically in ductal and lobuloalveolar cells (11). Thus, these locally acting growth factors provide excellent molecular markers to investigate the role of steroid receptors on ductal and lobuloalveolar development. First, however, it was necessary to establish whether E and P either alone or in combination could regulate the expression of Wnt-2 and Wnt-5B in a manner analogous to that observed during mammary gland development. BALB/c mice were treated with E and P to mimic the onset of pregnancy. RNA from the mammary glands of hormonally treated and untreated, ovx, and intact mice were examined for changes in gene expression using a quantitative, RT-PCR method (9). A decrease of approximately 4-fold relative to the untreated (time zero) group in Wnt-2 gene expression was observed after E and P treatment of intact BALB/c mice ($n = 3$, $P < 0.001$, Fig. 3A). A 2-fold decrease ($P < 0.002$) in Wnt-2 gene expression is observed after only 2 days of E and P treatment. Wnt-2 gene expression decreased progressively with daily E and P treatment and remained low to day 12 (data not shown). This decrease in gene expression of Wnt-2 is not, however, as dramatic (20-fold) as that observed after the onset of pregnancy (9).

Circulating E and P can cause cyclical repression and induction of PR expression levels and possibly attenuate the molecular effects of pharmacological doses of E and P. To eliminate the effects of endogenous ovarian hormones, three groups of ovx mice were implanted subcutaneously for 14 days with beeswax pellets that contained E and P together, E alone, or carrier. The thoracic mammary glands were collected from three animals at each time point within each treatment group at 1, 3, and 14 days after implantation of the pellets. A 3-fold decrease relative to the day 1 E and P treatment group was observed in Wnt-2 expression after 3 days, increasing to 5-fold at 14 days compared with control ($n = 3$, $P < 0.001$, Fig. 2B). Interestingly, mice treated with E alone showed the same 5-fold decrease relative to day 1 E alone mice in Wnt-2 gene expression after 14 days ($n = 3$, $P < 0.001$).

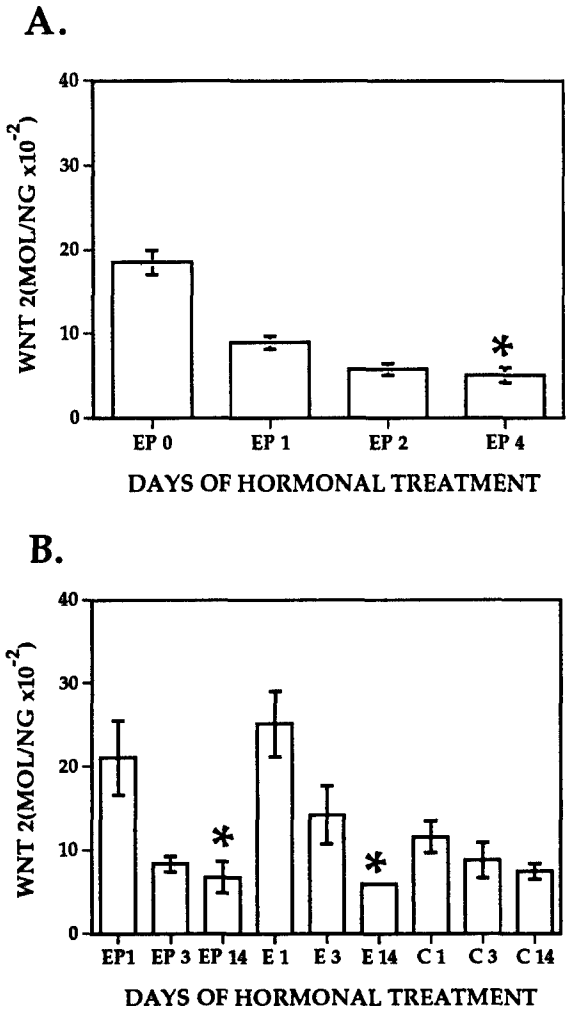

Fig. 3. The Response of Wnt-2 Gene Expression in Intact (A) and ovx (B) BALB/c Mice to E and P Treatment

A, Quantitative RT-PCR analysis of RNA from mammary glands of BALB/c mice injected daily with E and P, subcutaneously, for 8 days. EP refers to treatment with E and P for the number of days designated. Values: Molecules/nanogram RNA(MOL/NG) represent the mean ± SEM. The *star* denotes that EP 4 is statistically different from EP 0, $P < 0.001$, n = 3. B, Quantitative RT-PCR analysis of RNA from mammary glands of BALB/c mice treated with E and P, E, and vehicle beeswax implants for 1, 3, and 14 days. E refers to treatment with E alone for the number of days designated. The absolute values for the entire control group (C) were low in this experiment, possibly due to an effect of the vehicle, but did not change significantly with time. Values represent the mean ± SEM. The *stars* denote that EP 14 and E 14 are statistically different from EP 1 and E 1, respectively; $P < 0.001$, n = 3.

Wnt-5B Expression Is Induced by E and P in ovx and Intact Mice

During normal mammary gland development, Wnt-5B expression is observed initially at 6–8 weeks in the virgin mouse and increases at the onset of pregnancy with maximal expression observed at day 16–18 of pregnancy (9–11). Wnt-5B expression increased 4-fold by day 8 of E and P treatment of intact mice as compared with the untreated (time zero) mice and was maximally induced by day 16 ($P < 0.004$, n = 3) as illustrated in Fig. 4A. Thus, the increase in Wnt-5B gene expression, which parallels that observed during midpregnancy, requires chronic E and P treatment. The pattern of Wnt-5B expression in the ovx mice (Fig. 4B) was similar to that observed in the intact animal but displayed a more dramatic response. Wnt-5B expression remained low at day 1 and day 3 but increased 9-fold at day 14 relative to the day 1 E- and P-treated group ($P < 0.001$, n = 3). In contrast to the regulation of Wnt-2, there was no significant effect of E alone on Wnt-5B expression in ovx mice. The large increase observed in Wnt-5B expression in ovx mice

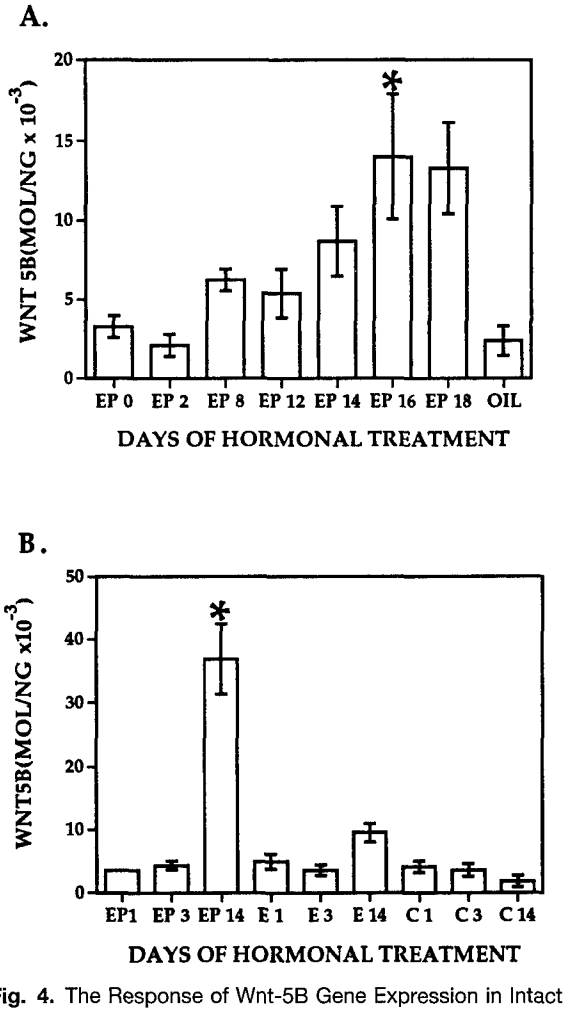

Fig. 4. The Response of Wnt-5B Gene Expression in Intact (A) and ovx (B) BALB/c Mice to E and P Treatment

A, Quantitative RT-PCR analysis of RNA from mammary glands of BALB/c mice injected daily with E and P, subcutaneously, for 18 days. Values represent mean ± SEM. The *star* denotes that EP 16 is statistically different from EP 0, $P < 0.004$, n = 3. B, Quantitative RT-PCR analysis of RNA from mammary glands of BALB/c mice treated with E and P beeswax implants for 1, 3, and 14 days. Values represent mean ± SEM. The *star* denotes that EP 14 is statistically different from EP 1; $P < 0.001$, n = 3.

may reflect the sensitization of the gland to P due to the absence of endogenous hormones and a rapid induction of PR gene expression.

Morphological Changes in Normal and Steroid Hormone-Treated Mice Correlate with the Changes in Wnt Gene Expression

Changes in Wnt-2 and Wnt-5B gene expression are coincident with morphological changes observed in the mammary gland in response to E and P treatment (Fig. 5). Mammary glands of 12-day E- and P-treated BALB/c mice (Fig. 5H) exhibit a morphology similar to that observed in an 8-day pregnant mouse. Several differences were observed, however, between the steroid hormone-treated glands and those from the normal midpregnant mouse, especially when comparing the first few days after hormone administration. For example, on the second day of treatment, transient alveolar proliferation was observed (Fig. 5F). However, by the fourth day these alveoli were no longer detectable, and a decrease in the amount of secondary branches was observed (Fig. 5). This transient alveolar budding has not been reported in mice treated with pharmacological doses of E and P but is similar to the effect observed in some strains of mice who respond to ovarian cycling by producing a transient alveolar proliferation in the mature virgin gland. This phenomena has, however, not been observed in BALB/c mice (1, 15). Surprisingly, at day 8 some major ducts displayed a ductal hypertrophy (Fig. 5G, *arrow*). This hypertrophy has been observed in mammary glands of mice implanted with hepatocyte-growth factor (HGF) and treated with E and P (16). Unlike the alveolar budding, this ductal hypertrophy was not transient and was still detectable in some glands at day 12–16 (Fig. 5H). Permanent alveoli appeared at day 12 and increased in number and density throughout the remainder of the treatment. This progression of morphological changes can be compared with the normal gland (1). During pregnancy, alveoli and secondary branching appear by day 4 and increase in density and number with the progression of pregnancy (Fig. 5, C and D).

These morphological alterations observed in hormonally treated and normal mammary glands can be correlated with the E- and P-induced changes in Wnt-2 and Wnt-5B gene expression. The appearance of transient alveoli at day 2 coincides with the decrease in Wnt-2 expression observed in E- and P-treated and normal glands. Conversely, permanent and functionally capable alveoli appear after 4–8 days of E and P treatment, preceding the increase in Wnt-5B expression. P concentrations increase gradually during pregnancy, affecting the formation of alveoli and the induction of the differentiated alveolar phenotype (6, 17). Appropriately, the increase in Wnt-5B expression in the E- and P-treated mouse requires

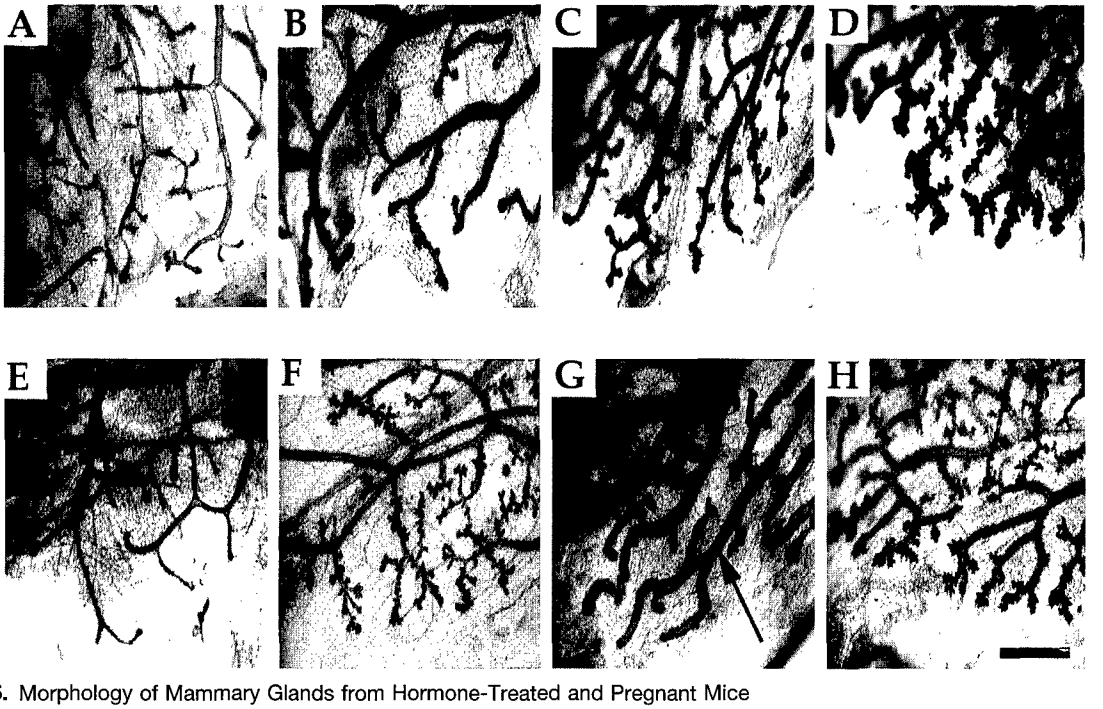

Fig. 5. Morphology of Mammary Glands from Hormone-Treated and Pregnant Mice

The progression of morphological changes in control mammary glands in response to the onset of pregnancy (panel A, 10-week virgin; B, day 2 pregnancy; C, day 4 pregnancy; D, day 12 pregnancy) and in E- and P-treated (panel E, untreated; panel F, E + P, 2 days of treatment; panel G, E + P, 4 days of treatment; panel H, E + P, 12 days of treatment). Mammary glands isolated from control and hormonally treated mice were stained with hematoxylin as described in *Materials and Methods*. Note the transient alveoli in panel F. The *arrow* in panel G denotes the hypertrophic duct. *Bar* = 100 μm.

long-term treatment of steroid hormones mimicking the pattern of morphological and gene expression changes, observed in the pregnant gland.

Differential Regulation of Wnt-2 and Wnt-5B Gene Expression in $PR^{-/-}$ Mammary Glands and in $PR^{-/-}$ Epithelium Transplanted into $PR^{+/+}$ Stroma after E and P Treatment

The response of Wnt-2 and Wnt-5B gene expression to the onset of pregnancy and exogenous E and P suggested that the P-signaling pathway might play a primary role in regulating Wnt gene expression in the mammary gland. To examine the role of the PR in Wnt gene regulation, Wnt gene expression levels were determined in $PR^{-/-}$ mice (12) after treatment with E and P. Wnt-5B gene expression did not change in response to E and P treatment in $PR^{-/-}$ mice (n = 3, Fig. 6A). However, the E and P repression of Wnt-2 expression was still observed but was not significant until day 8 of hormone treatment ($P < 0.003$, n = 3, Fig. 6B). This E-induced decrease in Wnt-2 gene ex-

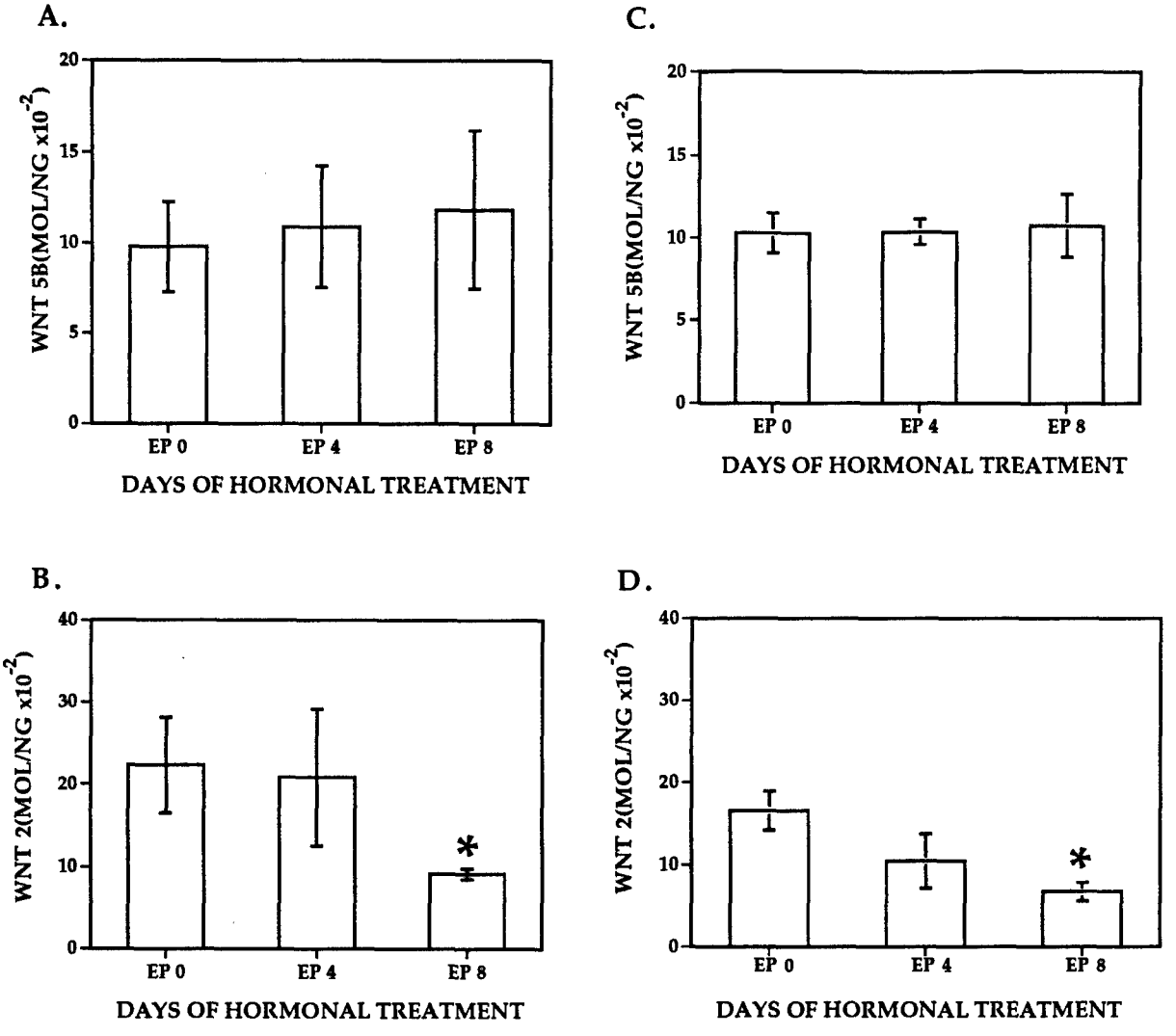

Fig. 6. The Response of Wnt Gene Expression in $PR^{-/-}$ Mammary Glands and in Transplanted $PR^{-/-}$ Epithelium into $PR^{+/+}$ Stroma to Steroid Hormone Treatment

A, Quantitative RT-PCR analysis of Wnt-5B gene expression from $PR^{-/-}$ mammary glands after daily injections with E and P, subcutaneously, for 0, 4, and 8 days (n = 3). B, Quantitative RT-PCR analysis of Wnt-2 gene expression from $PR^{-/-}$ mammary glands after daily injections with E and P, subcutaneously, for 0, 4, and 8 days. The *star* denotes that EP 8 is statistically different from EP 0; $P < 0.001$, n = 3. C and D, $PR^{-/-}$ epithelium was transplanted into cleared fat pads of $PR^{+/+}$ 129SvEv mice. After 10 weeks of growth, the mice were treated with E and P. C, Quantitative RT-PCR analysis of Wnt-5B gene expression in transplanted mammary glands after daily injections with E and P, subcutaneously, for 0, 4, and 8 days. Values represent the mean ± SEM (n = 6). D, Quantitative RT-PCR analysis of Wnt-2 gene expression in transplanted mammary glands after daily injections with E and P, subcutaneously., for 0, 4, and 8 days. Values represent the mean ± SEM. The *star* denotes that EP 8 is statistically different from EP 0; $P < 0.013$, n = 6.

pression was observed in the absence of any detectable changes in mammary gland morphology in the $PR^{-/-}$ mice.

To determine whether stromal or epithelial PRs were involved in differential Wnt gene response, the levels of Wnt transcripts were quantitated in RNA isolated from the previously described transplants. The role of the epithelium in the induction of Wnt-2 and Wnt-5B gene regulation was examined using $PR^{-/-}$ epithelium, transplanted into the stromal fat pad of cleared $PR^{+/+}$ hosts. In these transplants no increase in the level of P-dependent Wnt-5B expression was observed after 8 days of E and P treatment ($n = 6$, Fig. 6C). To confirm that the low level of Wnt-5B expression seen in the different samples was due to the response in the epithelium and not due to absence or degradation of RNA, the expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was also analyzed by RT-PCR. G3PDH transcripts are readily detectable and constant throughout the treatment period (data not shown). The same 129SvEv mice containing transplanted $PR^{-/-}$ epithelium and treated with E and P for 8 days still possessed the ability to repress Wnt-2 expression when subjected to steroid hormone treatment ($P < 0.013$, $n = 3$, Fig. 6D). Interestingly, the reduction of Wnt-2 gene expression was again delayed requiring 8 days of steroid hormone treatment as observed previously in $PR^{-/-}$ mice.

DISCUSSION

PR Deficiency in the Stroma and Epithelium Has Distinct Effects on Mammary Gland Development

The absence of lobuloalveolar development in the $PR^{-/-}$ transplants into the $PR^{+/+}$ fat pad after steroid hormone treatment indicates that the PR in the epithelium is required for this stage of mammary gland development. These results are consistent with previous studies showing that the absence of the PR influences lobuloalveolar development (12). Furthermore, in the reciprocal transplants, $PR^{+/+}$ epithelium into $PR^{-/-}$ stroma (Fig. 2, D and E), lobuloalveolar development was observed in response to E and P, indicating that the absence of the PR in the stroma does not significantly impair the ability of the epithelium to undergo alveolar differentiation. However, ductal growth was impaired, as the $PR^{+/+}$ epithelium failed to fill the $PR^{-/-}$ fat pad. In addition to the limited ductal development, unusual distended endbuds were present in these outgrowths. This restricted ductal growth may be due to disruption of reciprocal interactions between the stromal and epithelial compartments required for this stage of development. Less dramatic effects on ductal morphogenesis have been observed in the $PR^{-/-}$ mouse (12). This suggests that the $PR^{-/-}$ stroma, when recombined with $PR^{+/+}$ epithelium *in vivo*, lacks the mechanism to correctly interact with and stimulate growth in the epithelium and that the stroma fails to generate signals upon which the ductal growth of the epithelium is dependent. In contrast, in the $PR^{-/-}$ mouse, the $PR^{-/-}$ epithelium, in the presence of $PR^{-/-}$ stroma, has adapted to the absence of reciprocal signals, and the epithelium has become independent of these inputs required for ductal growth. This hormone-dependent reciprocity of growth regulation has been observed previously in recombination experiments with wild type and TfM androgen-insensitive mammary glands. These experiments revealed that the epithelium can induce the expression of mesenchymal androgen receptors. In turn, the mesenchyme condenses around the epithelium and causes an epithelial regression (18, 19).

The PR in the stroma is expressed in a temporally distinct pattern from the epithelial receptor, has a different signaling mechanism, and affects a separate group of target genes (5). Therefore, these data support the theory of two separate functional effects of mammary gland PR based on their compartmentalization and roles in development. The unexpected results observed in transplants of $PR^{+/+}$ epithelium in the $PR^{-/-}$ stroma suggest that there is a P-dependent stromal signal for ductal development. Recent *in vivo* studies of murine mammary glands treated with HGF in the presence of E and P suggest that HGF is a potential candidate second messenger for ductal growth in the mammary gland (16, 20). HGF-treated mammary glands respond to E and P by stimulating ductal growth. Interestingly, this growth factor has also been shown to regulate Wnt-5A expression (21). If this hypothesis is valid, it may be possible to rescue the $PR^{-/-}$ stroma defect by the direct addition of HGF. Unfortunately, because HGF knockouts are embryonic lethal and die before E16.5, no information has been obtained to date on mammary ductal development in these knockout mice (22).

Wnt-2 and Wnt-5B Gene Expression Are Regulated Independently by Steroid Hormones

This study demonstrates that two developmentally regulated Wnt genes are regulated by distinct mechanisms. The unique temporal and spatial patterns of expression of Wnt-2 and Wnt-5B suggest that these genes may play some role in the development of the mammary gland. The response of Wnt-2 and Wnt-5B to E and P treatment indicates that these genes are useful markers for the action of E in the virgin mammary gland, and for P during pregnancy, respectively. Wnt-2 gene expression is highest in the immature virgin gland of BALB/c mice and declines rapidly at the onset of pregnancy (9). In ovx and intact BALB/c mice this effect can be mimicked with the addition of pharmacological doses of E. This acute repression of Wnt-2 gene expression is correlated with the appearance of lobuloalveolar structures and the termination of ductal development. Conversely, an increase in Wnt-5B gene expression in ovx and intact mice requires chronic

treatment with E and P. Ovariectomy enhances the magnitude of this response. Without the stimulation from the ovaries, the basal levels of Wnt-5B expression are probably significantly reduced, thereby allowing an enhanced response. Wnt 5B provides one of the few molecular endpoints for the action of P, and changes in Wnt 5B are coincident with lobuloalveolar development.

The results from the $PR^{-/-}$ studies demonstrate that Wnt-5B gene expression is induced by P and dependent on the presence of PR specifically in the epithelial compartment. E alone has no effect on Wnt-5B expression. Interestingly, the PR present in the stroma cannot compensate for the absence of PR in the epithelium for lobuloalveolar development or for induction of Wnt 5B gene expression. This result implies that P acts directly on the epithelium to induce lobuloalveolar development and, either directly or indirectly, to activate Wnt-5B gene expression. E is required for induction and maintenance of PR expression in the mammary gland (6). Therefore, it is unlikely that the regulation of Wnt-5B expression is independent of E.

Wnt-2 gene expression was inhibited by administration of E in both $PR^{-/-}$ mice and in transplanted $PR^{-/-}$ epithelium. These results suggest that Wnt-2 gene expression is not primarily regulated by PR signaling. In the $PR^{-/-}$ studies there was a delay in the kinetics of the Wnt-2 response. The absence of the PR may have restricted the development of the gland in these mice and slowed the appearance of the ER, which normally appears at 4 weeks of age (23, 24). Alternatively, the absence of the PR could influence reciprocal interactions between the stroma and the epithelium, thereby preventing proper induction of ERs. ER gene expression is affected by feedback controls between E and other hormones including P (14).

Previous studies performed in Parks mice demonstrated Wnt-2 expression through midpregnancy and a repression of Wnt-2 and Wnt-5B expression after ovariectomy (11). Parks mice possess virgin lobuloalveolar development, which is absent in BALB/c mice, and it is possible that this epithelial sensitivity to estrous-associated hormones alters the regulation of Wnt-2 and Wnt-5B gene expression.

The rapid repression of Wnt-2 expression suggests the ER may be directly regulating Wnt-2. The ER is expressed in both the stroma and epithelium including the endbud (5, 14, 23–25). Interestingly, PR is induced by the ER-signaling pathway in the epithelial compartment 48 h after initial addition of E (26). This temporal delay in receptor response coincides exactly with the initial decrease observed in Wnt-2 gene expression after hormonal treatment. Therefore, the timing of this E-induced gene expression and localization of some Wnt-2 transcripts in the epithelium suggests that Wnt-2 could be regulated directly by E.

The Wnts Are Growth Factors with Pleiotropic Effects on Development

The development of the mammary gland is dependent on the interaction and cooperation of growth factors and hormones functioning through the stromal and epithelial compartments. Studies of PRL, epidermal growth factor, FGF, TGFα and β, insulin-like growth factor, and HGF action reveal that they are regulated in specific spatial and temporal patterns and have effects on proliferation and differentiation in mammary gland development (1, 27–30). The developmentally associated expression pattern, their role in the development of other organisms, biochemical characteristics, and hormonal regulation of the Wnts suggest that they are members of this complex family of locally acting growth factors.

The function of the Wnt genes in the development of the mammary gland can only be inferred from limited expression studies *in vivo* and *in vitro* and functional studies in other organisms. Wingless, the *Drosophila* homolog of Wnt-1, has proliferative, inductive, and cell fate determination functions (8). In addition, Wnt genes have demonstrated functional roles in *Xenopus*, mouse, and chicken (31–36). These diverse studies revealed that Wnt genes can possess inductive, growth-stimulatory, and growth-restrictive functions all within a single organism.

In the mammary gland, overexpression of Wnt-1 influences the proliferation of mammary epithelium (37). The expression of Wnt-4 and Wnt-5A has been inversely correlated with proliferation in mammary epithelial cells (38). Because of its localization both within and around the highly proliferative terminal endbud, it is possible that Wnt-2 has a role in regulating proliferation in the virgin gland. The pattern of Wnt-5B expression and its dependence on the PR suggests that it interacts with cells in a more differentiated state. Interestingly, proliferation is high in the pregnant gland coincident with the increase in Wnt-5B expression. Localization of Wnt-5B transcripts to the ductal epithelium reveals it is expressed in the proper cellular location to be involved in regulating proliferation in these cells. The localization of Wnt-5B and Wnt-2 transcripts to the ductal epithelial and stromal compartments of the mammary gland (9, 11), respectively, suggest that although these two genes may have separate or even overlapping functional roles, their temporal and spatial expression patterns restrict their activity to specific stages of development. Therefore, it is probable that the expression of these Wnt genes is regulated in a specific manner to restrict their functional activities to particular developmental stages in the mammary gland.

Alteration of Wnt Gene Expression Can Transform Mammary Epithelium

In the mammary gland, ectopic expression of the Wnt genes has dramatic consequences on the transforma-

tion and development of the gland. Inappropriate expression of Wnts either temporally or spatially may result in mammary tumorigenesis. For example, Wnt-1 and Wnt-4 have been demonstrated to affect the development and transformation of the gland *in vivo* (37, 39, 40). Numerous other Wnts, including Wnt-2 and Wnt-5B, have *in vitro* transforming effects (41, 42). These *in vitro* transfection experiments have revealed that separate classes of Wnts exist that are distinguished by their transforming ability (43), although the properties defined in these *in vitro* assays do not always correspond to their effects *in vivo* (R. C. Humphreys and J. M. Rosen, submitted for publication).

In addition, overexpression of Wnt genes, including Wnt-2 and Wnt-5B, has been found associated with tumors in the breast and intestinal epithelium (44–47). Thus, loss of regulatory control on these two Wnt genes, as with other growth factor molecules like TGF-β and FGF (48, 49), has deleterious consequences for the development of the mammary gland. Interestingly, compartment switching of Wnt-2 expression from breast fibroblasts to tumor epithelium has been observed recently in human breast tumors (50). Therefore, there is evidence for a critical role of Wnt-2, and possibly Wnt-5B, in the transformation of the gland. Since most of the Wnt knockouts are embryonic lethals resulting in neural or kidney defects, the precise functional roles of these and other Wnt family members on normal mammary gland development will require the use of tissue-specific or regulated knockouts.

To summarize, the Wnt genes act in a cell-autonomous manner in cooperation with other growth factors and have pleuripotent effects on various developmental processes within the same organism (8). Wnt gene expression can be differentially regulated by steroid hormones in the mammary stroma and epithelium where they may act as locally acting growth factors to influence ductal and lobuloalveolar development. Hopefully, with the recent discovery of the Wnt receptor in *Drosophila* (51), the mechanism of Wnt action and the function of the individual Wnt family members in mammary gland development will begin to be illuminated.

MATERIALS AND METHODS

Animals

BALB/c mice were acquired from Charles River Laboratories (Wilmington, MA) or from a breeding colony at Baylor College of Medicine, courtesy of Dr. Daniel Medina. Mice carrying the PRKO mutation in the 129SvEv inbred genetic background were used in these studies. All animals were maintained according to IACUC approved guidelines.

Isolation of Mammary Glands and RNA

Number 4 (thoracic) mammary glands were removed from aged matched 6- and 8-week-old virgin BALB/c and C3H mice using standard surgical techniques. The thoracic and inguinal mammary glands from 11-week-old wild type control mice, 6-week-old 129SvEv $PR^{-/-}$ mice, 129SvEv $PR^{+/+}$, and 129SvEv $PR^{-/-}$ mice with transplanted PR-deficient epithelium or wild type epithelium, respectively, were removed using standard surgical techniques. For morphological analysis, mammary glands were fixed in Tellyesniczky's solution for 5 h and stained with hematoxylin as described previously (52). For isolation of RNA, mammary glands were homogenized in a PT2000 Polytron (Brinkmann, Westbury, NY) with RNazol (Biotecx, Houston, TX) as described by the manufacturer or homogenized in 4 M guanididium isothiocynate (Sigma, St. Louis MO) and isolated by CsCl centrifugation method. RNA was quantitated spectrophotometrically and stored at −20 C in 70% ethanol.

Construction and Transcription of cRNA Templates

Quantitative noncompetitive RT-PCR was performed as previously described (9). Complementary DNAs from Wnt-5B and Wnt-2 were prepared according to standard bacterial plasmid isolation protocols, and the DNA was purified on Qiagen (Qiagen, Chatsworth, CA) columns according to the manufacturer and isolated from the vector using unique restriction enzymes. To construct the Wnt-2 cDNA deletion template, *Sty*I (New England Biolabs, Beverly, MA) was used to excise a fragment from bases 493–580. Digestion products were separated from the small internal fragment, religated, and subcloned into the vector pBKSII (Stratagene, La Jolla, CA). Clones were analyzed for a size difference and sequenced to confirm the location of the deletion. The Wnt-5B template was constructed in the same manner with an *Ava*I (New England Biolabs, Beverly MA) deletion of bases 501–576. Both templates were sequenced to confirm the orientation in the vector and the presence of an internal deletion. These constructs were used as templates for *in vitro* transcription reactions as described in Promega Protocols and Applications Guide, ed 2 (Promega, Madison WI). The cRNA reactions were treated with 1 U of ribonuclease-free RQ1 deoxyribonuclease in deoxyribonuclease buffer (Promega) for 60 min at 37 C and then extracted with phenol-chloroform twice and precipitated with 3 M NaAc and 100% ethanol at −20 C. The cRNA was resuspended in Tris-EDTA, quantitated spectrophotometrically, and stored at −20 C in 70% ethanol. Each template was assayed by PCR to confirm the absence of contaminating cDNA template. Optimum RT-PCR conditions for each of the templates were developed that allowed a linear response with respect to the RNA input and exhibited noncompetitive PCR.

Quantitative RT-PCR

Isolated RNA was transcribed in a reaction consisting of 1× *Taq* polymerase buffer (Promega), 3 mM $MgCl_2$, 100 pmol hexanucleotide random primers (Boehringer Mannheim, Indianapolis, IN) 1.25 U of RT (GIBCO BRL, Gaithersburg MD), 1 mM of each of four deoxynucleoside triphosphates (Pharmacia, Milwaukee WI), and 20 U of RNasin (Pharmacia, Milwaukee WI) in a final reaction volume of 20 μl. Fifty nanograms, 100 ng, and 150 ng of sample RNA were added to separate RT reactions. A constant amount of cRNA template (~10,000 molecules) was added to each RT reaction as an internal standard to control for differences in RT and PCR reaction efficiency.

The primer sequences for the Wnt-2 and Wnt-5B amplifications, respectively, were:

forward: 5′-AGTCGGGAATCGGCCTTTGTTTACG-3′ and reverse: 5′-AAAGTTCTTCGCGAAATGTCGGAAG-3′; forward: 5′-GACAGCGCCGCGGCCATGCGC-3′ and reverse: 5′-CATTTGCAGGCGACATCAGC-3′. PCR conditions were 94 C for 1 min, 60 C for 2 min, and 72 C for 3 min, for 30 cycles and 94 C for 1 min, 65 C for 2 min, and 72 C for 3 min

for 32 cycles for Wnt-2 and Wnt-5B, respectively. Primers for G3PDH were: forward: 5′-AGAGGCCTTTGCTCGAACTG-GAAAG-3′ and reverse: 5′-CACCAAGACGTCTGTCGC-CTACTTA-3. PCR conditions were 94 C for 1 min, 60 C for 2 min, and 72 C for 3 min, for 30 cycles. All PCRs were followed by an extension at 72 C for 5 min. PCR was performed with 10 μl of each RT reaction, 2 mM magnesium chloride, 1×PCR buffer (Promega), 0.1 μCi [α-^{32}P]dCTP (NEN DuPont, Boston, MA), 1 U of *Taq* polymerase (Promega, Madison WI) in a final reaction volume of 50 μl. Ten microliters of the RT-PCR products were separated on a 2% Nusieve agarose (FMC Bioproducts, Rockland, ME) gel and transferred overnight in 0.4 M NaOH to Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK), and the radioactive signal was quantitated with 4–8 h exposure on a PhospholImager (Molecular Dynamics, Sunnyvale, CA).

Steroid Hormone Treatment of Mice

All groups of mice were treated with 1 mg of P (Steris, Phoenix, AZ) and 1 μg of 17 β-estradiol (Sigma) per day in 60 μl of sesame seed oil (Sigma) subcutaneously. Mammary glands were collected at days 0, 1, 2, 4, 8, 12, and 18. Animals were ovariectomized and allowed to regress for 4 weeks before hormone treatments were begun. Beeswax implants containing 20 μg of E and/or 20 mg of P were synthesized by adding the powdered form of the hormones to melted beeswax. The suspended hormone mixture was dropped onto dry ice to form pellets. The pellets, synthesized to deliver 1 mg P and 1 μg E/day, respectively, were implanted subcutaneously in the neck of the mice for 2 weeks. Inguinal mammary glands were collected at days 1, 3, and 14 and analyzed as described.

Transplantation Studies

Tissue fragments of 10-week-old virgin $PR^{-/-}$ mammary epithelium were isolated and implanted into six PR-positive 129SvEv hosts using the technique described by DeOme *et al.* (13). Epithelium from 129SvEv hosts was removed as described (13). In addition, tissue fragments from a 10-day pregnant 129SvEv $PR^{+/+}$ mammary epithelium were isolated and implanted into four $PR^{-/-}$ 129SvEv hosts. Due to the limited number of $PR^{-/-}$ homozygote recipients and the limited extent of ductal outgrowth, these glands could not be examined for changes in Wnt gene expression. Mammary gland epithelial transplants were allowed to proliferate and penetrate the stromal fat pad for 10 weeks and then treated with steroid hormones as described above. Control experiments with wild type 129SvEv $PR^{+/+}$ epithelium into cleared fat pads of three wild type 129SvEv $PR^{+/+}$ mice were performed in the same manner.

Whole Mount Staining and Sectioning

The whole gland staining was carried out essentially as described (25) except that glands were stained for only 2 h in hematoxylin.

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